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(54) **Biotin biosynthesis in bacillus subtilis.**

(57) The present invention is directed to DNA sequences of genes that encode a biotin biosynthetic enzyme of *B. subtilis* or of a closely related species thereof, vectors comprising such DNA sequences, cells comprising such DNA sequences and vectors and a process for the production of biotin by such cells.

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The present invention relates to the production of biotin using a genetically engineered organism.

Biotin (vitamin B₇ or vitamin H), a coenzyme for carboxylation and decarboxylation reactions, is an essential metabolite for living cells. Exogenous biotin is required for most higher organisms; however many bacteria synthesize their own biotin.

5 The enzymatic steps involved in the biotin synthetic pathway from pimelyl-CoA (PmCoA) to biotin have been elucidated in *Escherichia coli* and *Bacillus sphaericus* [Fig. 1; reviewed in Perkins and Pero, *Bacillus subtilis and other Gram-Positive Bacteria*, ed. Sonenshein, Hoch, and Losick, Amer. Soc. of Microbiology, pp. 325-329 (1993)]. The steps include the conversions of 1) pimelyl-CoA to 7-keto-8-amino pelargonic acid (7-KAP or KAPA) by 7-KAP synthetase (*bioF*); 2) 7-KAP to 7,8-diamino-pelargonic acid (DAPA) by DAPA aminotransferase (*bioA*); 3) DAPA to dethiobiotin (DTB) by DTB synthetase (*bioD*); and 4) DTB to biotin by biotin synthetase (*bioB*). Synthesis of PmCoA reportedly involves different enzymatic steps in different microorganisms. The *E. coli* genes involved in steps preceding pimelyl-CoA synthesis include *bioC* [Otsuka et al., *J. Biol. Chem.* 263, 19577-19585 (1988)] and *bioH* [O'Regan et al., *Nucleic Acids Res.* 17, 8004 (1989)]. In *B. sphaericus*, two different genes, *bioX* and *bioW*, are thought to be involved in PmCoA synthesis. *BioX* is thought to be involved in pimelate biosynthesis [Gloeckler et al., *Gene* 87, 63-70 (1990)], and *bioW* has been shown to encode pimelyl-CoA synthetase which converts pimelic acid (PmA) to PmCoA [Ploux et al., *Biochem. J.* 287, 685-690 (1992)]. Neither *B. sphaericus* gene, *bioW* or *bioX*, has significant sequence similarity with the *E. coli bioC* and *bioH* genes either at the nucleotide or protein level [Gloeckler et al. (1990) *supra*].

20 In *E. coli*, the biotin biosynthetic genes are located in three or more operons in the chromosome. The *bioA* gene is located in one operon and the *bioBFCD* genes are located in a second closely linked operon. The *bioH* gene is unlinked to the other *bio* genes (Fig. 2; Eisenberg, M.A. 1987 in *Escherichia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology, vol. 1, Amer. Soc. Micro. Wash. D.C.).

25 In *B. sphaericus* the organization of the *bio* genes is clearly different from that in *E. coli*. Gloeckler et al. [(1990) *supra*] have isolated and characterized two unlinked DNA fragments from *B. sphaericus* that encode *bio* genes. One fragment contains an operon encoding the *bioD*, *bioA*, *bioY*, and *bioB* genes, and the other fragment contains an operon encoding the *bioX*, *bioW* and *bioF* genes (Fig. 2). The order and clustering of *bio* genes is different in *E. coli* and *B. sphaericus* (Fig. 2).

30 Fisher (U.S. Patent 5,110,731) provides a system for producing biotin wherein the genes of the biotin operon of *E. coli* are transformed into, and expressed in, a retention-deficient strain of *E. coli*.

Gloeckler et al. (U.S. Patent 5,096,823) describes genes involved in the biosynthesis of biotin in *B. sphaericus*: *bioA*, *bioD*, *bioF*, *bioC*, and *bioH*. *B. sphaericus* genes for *bioA* and *bioD* were cloned into both *E. coli* and *B. subtilis*. The *bioA* and *bioD* genes were stably integrated into *B. subtilis* Bio⁻ auxotrophs, and prototrophic strains were selected.

35 British Patent 2,216,530 provides plasmids containing gene(s) for *E. coli bioA*, *bioB*, *bioC*, *bioD*, and *bioF* isolated from other *E. coli* genetic material, e.g., control sequences. The plasmids are capable of replicating and being expressed in non-*E. coli* strains, preferably in yeast.

Three biotin synthesis deficient mutants of *B. subtilis* (*bioA*, *bioB*, and a gene termed *bio112* which may be analogous to *E. coli bioF*) have been reported [Pai, *Jour. Bact.* 121, 1-8 (1975); and Gloeckler et al. (1990) *supra*].

40 Nippon Zeon Co. Ltd. (U.S. Patent 4,563,426) discloses biotin fermentation that includes adding pimelic acid after culturing for about 24 hours. Transgene SA and Nippon Zeon Co. Ltd. (European Patent Application Publication No. 379 428) disclose adding pimelic acid to a biotin fermentation medium.

45 The invention generally provides the genes of the biotin synthetic operon of *B. subtilis* and closely related species to be used for high level production of biotin. Specific aspects of the invention are described in greater detail below. We have specifically identified, cloned, and engineered a previously unknown gene (*bioI*), which encodes a cytochrome P-450-like enzyme. We have also developed a strategy to overexpress the entire *B. subtilis bio* operon (which, when engineered with a strong promoter, is unexpectedly toxic to *E. coli*) by cloning two *bio* operon fragments separately, combining them *in vitro*, and transforming the host organism with the resulting ligated construction. Cloning the two fragments was further complicated by difficulty obtaining the 5' end of the operon, due to toxicity in *E. coli*. The invention particularly features the full-length operon obtained by the above strategy. These and other features of the invention are described in greater detail below.

55 In one aspect, therefore, the invention features a DNA comprising a DNA sequence selected from the group consisting of: (a) a DNA sequence of a gene that encodes a biotin biosynthetic enzyme of *Bacillus subtilis*, or of a species closely related to *Bacillus subtilis*, (b) a DNA sequence that encodes a biologically active fragment of (a); or (c) a DNA sequence that is substantially homologous to (a) or (b). Also, as used herein, a species which is "closely related" to *B. subtilis* includes a member of a cluster of *Bacillus* spp.

represented by *B. subtilis*. The cluster includes, e.g., *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. amyloliquefaciens*, *B. megaterium*, *B. cereus* and *B. thuringiensis*. The members of the *B. subtilis* cluster are genetically and metabolically divergent from the more distantly related *Bacillus* spp. of clusters represented by *B. sphaericus* and *B. stearothermophilus* [Fig. 3, Priest, in *Bacillus subtilis and other Gram-Positive Bacteria*, supra pp. 3-16; Stackebrandt, et al. *J. Gen. Micro.* 133, 2523-2529 (1987)].

As noted above, we have discovered a novel gene (*bioI*) present in *B. subtilis* and closely related species thereof, which is particularly important to deregulated production of biotin, and that gene is included in the DNA of preferred embodiments of the first aspect of the invention. Also preferably, at least *bioA* and *bioB* are included in the DNA of the first aspect. *BioD*, *bioF*, *bioW*, and ORF2 (encoding a β -keto reductase-like enzyme) may also be advantageously included in the DNA of the invention. At least two of the above-defined genes may be included in the DNA. The DNA sequences may be operably linked to a transcriptional promoter, e.g., a constitutive promoter such as a promoter derived from the SP01 bacteriophage. The entire biotin operon of *Bacillus subtilis*, or a closely related species thereof, may be linked to a single transcriptional promoter. Moreover, we have learned that it is particularly useful to include a second promoter i.e., one or more of the DNA sequences is/are operably linked to a first transcriptional promoter, and at least a second one of the genes is operably linked to a second transcriptional promoter. The first promoter may be operably linked to one or more of *bioA*, *bioB*, *bioD*, *bioF*, and *bioW* of *B. subtilis*, or a closely related species thereof. The other promoter may be operably linked to one or more of *bioI*, *bioA*, *bioB*, or a combination thereof. In a particularly preferred embodiment, the first promoter controls transcription of the entire operon, and transcription of *bioI*, optionally with *bioA* and/or *bioB*, is also controlled by the second promoter. The DNA may include a mutated regulatory site of a biotin operon of *B. subtilis* or a closely related species, such as an operator, a promoter, a site of transcription termination, a site of mRNA processing, a ribosome binding site, or a site of catabolite repression. By mutation, we mean an insertion, a substitution, or a deletion with respect to the wild type regulatory site.

A second aspect of the invention relates to our discovery of *Bacillus subtilis bioI*. This aspect features that gene or a gene specifically hybridizable to *Bacillus subtilis bioI*. It also features a biotin biosynthetic enzyme encoded by such a gene.

The invention also features vectors comprising such DNAs as described above and cells comprising such a vector or such DNAs. Preferably, the DNA is amplified to multiple copies in such cells. Also preferably, the DNA is stably integrated into the chromosome of the cell. The DNA may be integrated at multiple sites in the chromosome, at least one of which is the *bio* locus, and in multiple copies at each such site. Also preferably, the cell is characterized by a mutation that deregulates production of biotin or a biotin precursor, in addition to the presence of the DNA. Such mutated cells produce an increase in biotin in comparison to wild-type cells lacking the DNA. Such a mutation may be one that confers resistance to azelaic acid and/or it may be a mutation in *birA*.

The above described cells are used in methods of producing biotin or a precursor thereof in which the cells are cultured for a time and under conditions which allow synthesis of biotin or the precursor, and biotin or precursor is then isolated, preferably from the extracellular media of the cell.

Yet another aspect of the invention features a recombinant protein encoded by a DNA as described above or a recombinant biotin biosynthetic enzyme comprising an amino acid sequence that is substantially homologous to the amino acid sequence of a biotin biosynthetic enzyme of *Bacillus subtilis*, or a closely related species thereof.

A final aspect of the invention features a method of selecting a mutant *Bacillus subtilis* cell characterized in being deregulated for biotin production, by: (a) providing a population of *Bacillus subtilis* cell; (b) allowing the population to reproduce in the presence of azelaic acid; (c) selecting a cell that is resistant to azelaic acid; and (d) screening the cell, or a daughter cell further mutated to deregulate biotin production, for the ability to overproduce biotin.

The above description of the invention may be further understood by reference to the following definitions and explanations. The vector DNA may include a DNA sequence which is substantially homologous to a gene (or to a DNA sequence that encodes a biologically active fragment of the gene) that encodes a biotin biosynthetic enzyme of *Bacillus subtilis*, or a closely related species thereof. The DNA sequence diverges from the wild type sequence by including a mutation, e.g., a deletion, an insertion, or a point mutation, that enhances the synthesis of biotin when the DNA sequence is expressed in a cell. At least two, three, four, five, or six, or preferably all, of the biotin operon genes, may be operably linked to a transcriptional promoter to yield a messenger RNA. As used herein, "operon" refers to one or more genes co-transcribed from the same promoter. "Biotin operon" refers to a group of genes whose gene products are involved in an aspect of biotin biosynthesis. By "promoter" is meant a nucleic acid sequence recognized by an RNA polymerase enzyme that initiates transcription of a gene located in the 3' direction of

the promoter to yield a messenger RNA. By "operably linked to a transcriptional promoter" is meant that the gene is sufficiently proximal to the promoter for an RNA transcript initiated at the promoter to include messenger RNA that is complementary to that gene. The transcriptional promoter is either a constitutive promoter, e.g., a promoter derived from the SP01 bacteriophage, or an inducible promoter.

Any of the genes of the operon can include a mutation that enhances the synthesis of biotin when the DNA sequence is expressed in a cell. In a related embodiment, a regulatory site in the biotin operon, or in a gene of the biotin operon, can be altered, e.g., by mutation, so as to increase the level of biotin produced in a cell. Examples of regulatory sites that can be altered include a site of transcription termination, an operator site, a site of mRNA processing, a ribosome binding site, or a site of catabolite repression.

Any of the vectors of the invention can be included in a host cell. The preferred host cell is a *B. subtilis* cell for the reasons discussed below. However, the vectors of the invention can also be inserted into another type of host cell, e.g., an *E. coli* cell, or any host cell containing the apparatus necessary to maintain the vector and/or to express a gene of the biotin operon located on the vector. Where the host cell is used for expression, it is also desirable for the host cell to have the ability to secrete biotin into the extracellular medium, as does *B. subtilis*, simplifying collection of the biotin product. Some of the host cells that can be used include, but are not limited to, Gram-positive bacteria, e.g., *B. subtilis* cells such as *B. subtilis* 168, W23, or *natto* strains, other *Bacillus* strains such as *B. licheniformis*, *B. amyloliquefaciens*, *B. pumilus*, *B. megaterium*, or *B. cereus* [for *Bacillus* strains see "Bacillus Genetic Stock Center Catalogue of Strains", Ohio State Univ. Depart. Biochem., Columbus, OH, edited by D. H. Dean (1986) 3rd edition], or other Gram-positive cells such as *Lactococcus*, *Lactobacillus*, *Corynebacterium*, *Brevibacterium*, *Staphylococcus*, *Streptomyces*, or *Clostridium*; Gram-negative bacteria, e.g., strains of *E. coli*, *Salmonella*, *Serratia*, or *Klebsiella*; or fungal cells, e.g., yeast. For strains and vectors useful with these Gram-positive cells see "Bacillus subtilis and other Gram-Positive Bacteria", [edited by Sonenshein, Hoch, and Losick, Amer. Soc. of Microbiology (1993)]. Biotin genes from Gram-positive organisms can be expressed in Gram-negative bacteria [Gloeckler et al., supra] and even fungal genes from *Saccharomyces cerevisiae* have been expressed in *E. coli* [Zhang et al., Archives of Biochemistry and Biophysics 309, 29-35 (1994)].

Where the vector is an extrachromosomal element it can be amplified, i.e., to multiple copies, in the cell. Alternatively, if the vector is not an extrachromosomal element, the vector can be stably integrated into the chromosome of the cell. Integrated vectors also can be amplified to multiple copies in the cell, i.e., integration can occur at multiple sites, or in multiple copies at each site. Integration may occur at a random site on the chromosome, or preferably integration is directed to a preferred chromosomal locus, e.g., the *bio* locus. The whole vector can integrate into the chromosome, or only the biotin biosynthetic sequences themselves can be integrated into the chromosome absent at least a portion of the non-biotin biosynthetic sequences, e.g., the replicon sequences. The cell containing the vector or biotin operon sequences can further be deregulated for biotin production.

By "deregulated for biotin production" is meant that a negative limitation that controls the level of biotin biosynthesis has been at least partially removed from the cell. A negative limitation includes, but is not limited to a regulatory protein (e.g., a repressor), a site of action of a regulatory protein (e.g., an operator), inhibitory factor, or a low level of a rate limiting enzyme. The cell can include a mutation in a genetic locus that complements the *birA* locus of *E. coli*. Examples of *B. subtilis* strains that include a mutation that causes an increase in biotin secretion include, but are not limited to, the strains HB3, HB9, HB15, HB43, α -DB9, α -DB12, α -DB16, and α -DB17, or any of the mutant or engineered strains listed in Table 8, Table 9, Table 10. Biotin is preferably secreted into the extracellular media to a concentration of at least 0.1 mg/l, 1 mg/l, 10 mg/l, 100 mg/l, 300 mg/l, 500 mg/l, 750 mg/l, or 1.0 g/l. Preferably, the host cell is *B. subtilis*, but it can also be any of the above-listed host strains. By "vitamer" or "biotin vitamer" is meant any of the compounds preceding biotin in the biosynthetic pathway that can be used to feed yeast, e.g., the following compounds shown in Fig. 1: 7-KAP, DAPA, or desthiobiotin. The term "biotin precursor" includes each of the biotin vitamers listed above, as well as PmA and PmCoA. By substantially homologous we mean having sufficient homology to yield specific hybridization under conditions that allow hybridization to DNA of *Bacillus* species within the cluster that includes *B. subtilis*, but are too stringent to allow hybridization to DNA of organisms outside the cluster of species closely related to *B. subtilis*. Suitable probes for this purpose are provided below. Suitable hybridizations use relatively stringent conditions. For example: nitrocellulose filters containing denatured DNA are incubated with a radioactively labeled DNA or RNA probe in the presence of 5XSSC (0.75M NaCl and 0.075M Na citrate, pH 7.0), 10-50% formamide, 1X Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% pyrrolidone), and 100 μ g/ml denatured salmon sperm DNA at 37-42°C. Those skilled in the art will understand that stringency can be gradually increased (e.g., by increasing formamide concentration or temperature) until suitable specificity is obtained (i.e., non-specific binding is reduced or eliminated).

By "biotin synthetic enzyme" is meant any one of the enzymes that form the biotin biosynthetic pathway as shown in Fig. 1, or discussed herein, as well as enzymes encoded by genes newly disclosed herein, e.g., *biol*, or ORF2. The term "biotin biosynthetic enzyme" also includes a portion or fragment of a native biotin biosynthetic enzyme that performs the biochemical function of a biotin biosynthetic enzyme of *B. subtilis*. The size of such a portion or fragment of a biotin biosynthetic enzyme is determined by the functional requirement that it retain the biochemical activity of the native enzyme. There are many examples in the literature of enzymes that retain one or more activities after shortening of the polypeptide chain by proteolysis or by truncating the associated gene [for example, see Dautry-Varsat and Cohen, *J. Biol. Chem.* 252, 7685-7689 (1977)].

As used herein, the term "fragment" or "portion", as applied to a polypeptide, will ordinarily be at least about 10 contiguous amino acids, typically at least about 20 contiguous amino acids, usually at least about 30 contiguous amino acids, preferably at least 50 contiguous amino acids, and most preferably at least about 60 to 80 contiguous amino acids in length. Similarly, by the term "fragment" in the context of a nucleic acid is meant a DNA sequence that encodes a polypeptide fragment as defined above. The ability of a candidate fragment to perform the biological activity of the corresponding naturally-occurring enzyme can be assessed by methods known to those skilled in the art, including, but not limited to, the following protocols: Assays of pimelyl-CoA synthetase (*bioW*), 7-KAP synthetase (*bioF*), DAPA aminotransferase (*bioA*), and DTB synthetase (*bioD*) are described by Izumi et al. [*Methods in Enzy.* 62, 326-338 (1979)]. A cell-free assay of biotin synthetase (*bioB*) is described by Ituku et al. [*Biosci. Biotech. Biochem.* 56, 1780-1785, (1992)]. The product of *biol* may be characterized as a cytochrome P-450 by the spectral determinations described by Omura et al. [*J. Biol. Chem.* 239, 2310-2378 (1964)].

By "recombinant" is meant that the gene encoding the enzyme has been removed from its naturally occurring site in the *B. subtilis* chromosome and inserted, either permanently or transiently, into a vector by techniques of genetic engineering known to one skilled in the art. Preferably, the vector includes sequences allowing for the expression of the inserted gene.

A "vector" as used herein refers to a nucleic acid molecule that can be introduced into a cell, e.g., by transfection, by transformation, or by transduction. Vectors include, but are not limited to, plasmids, bacteriophages, phagemids, cosmids, and transposons. Examples of vectors for use herein include, but are not limited to, pBR322, pCL1920, pCL1921, pUC18, pUC19, or pSC101. These vectors replicate in *E. coli* and other bacteria but do not replicate in *B. subtilis*, and are thus useful as integration vectors in *B. subtilis* after addition of an appropriate selectable marker. Other vectors commonly used in *B. subtilis* included the plasmids pUB110, pE194, pC194, and their derivatives which replicate in *B. subtilis*, or any of the integrational vectors, plasmids, temperate phage vectors or transposons described in Chapters 40-44 of *Bacillus subtilis and Other Gram-Positive Bacteria*, (*supra*, pp. 585-650). Additional vectors used in numerous microorganisms are described in "Cloning Vectors: A Laboratory Manual" [Pouwels et al. Elsevier (1985) with supplementary updates in 1986 and 1988]. Recombinant, engineered *B. subtilis* DNA may also be inserted (by homologous recombination) and amplified in the *B. subtilis* chromosome without using any replicating plasmid vectors.

A "substantially pure nucleic acid," as used herein, refers to a nucleic acid sequence, segment, or fragment that has been purified or separated from the sequences which flank it in its naturally occurring state, e.g., a DNA fragment that has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components that naturally accompany it in the cell. Preferably, the "sequence encoding a biotin biosynthetic enzyme of *B. subtilis*" is a major component of the total purified nucleic acid sequence, e.g., at least 1% or 10% of the total nucleic acid sequence.

"Homologous," as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA or RNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomer, subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. A "best-fit" homology can be achieved by adjusting the alignment of the sequences. The homology between two sequences is a function of the number of matching or homologous positions, e.g., if half (e.g. 5 positions in a polymer 10 subunits in length), of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. There may be gaps of non-homologous sequences among homologous sequences. "Substantially homologous" sequences are those that differ one from the other only by conservative substitutions. For example, where the substitution is in a nucleic acid sequence, the substitution either does not cause a change in amino acid at that position, or the substitution

results in a conservative amino acid substitution. A "conservative amino acid substitution" is, for example, a substitution of one amino acid for another of the same class (e.g., amino acids that share characteristics of hydrophobicity, charge, pK_a , or other conformational or chemical properties, e.g., valine for leucine, arginine for lysine) or by one or more non-conservative amino acid substitutions, deletions, or insertions, located at positions of the amino acid sequence that do not destroy the biological activity of the polypeptide (as described above). An amino acid sequence is included within the scope of the invention if it differs by a modification that reduces or alters the biological activity of one domain of a multiple-domain enzyme, while preserving a second biological activity in a second domain of the enzyme. Generally, a polypeptide is considered to be within the scope of this invention if it is at least 75%, preferably at least 80%, or most preferably at least 90%, homologous to the naturally occurring amino acid sequence of a biotin biosynthetic enzyme of *B. subtilis*. A nucleic acid sequence is considered to be within the scope of this invention if it is at least 70%, preferably at least 80%, or most preferably at least 90%, homologous to a naturally occurring nucleic acid sequence encoding a biotin biosynthetic enzyme of *B. subtilis*.

Bacterial strains containing the *B. subtilis* genes or DNA sequences provided herein are useful for producing high levels of biotin or of a biotin precursor, these compounds being useful in turn as a dietary additive for humans or animals. For instance, biotin can be supplied to a domesticated animal, e.g., a cow, chicken, or a pig, as an additive to a commercial preparation of animal feed. In addition, biotin can be added to a vitamin dietary supplement for human use. Biotin is also useful as a reagent for research and diagnostic procedures. For example, biotin is used as a non-radioactive label for proteins and nucleic acids. Biotin-labelled proteins are detectable by virtue of biotin's naturally occurring ability to bind to avidin, a protein found in egg-white, or to streptavidin, a biotin-binding protein produced by a streptomycete.

In the following paragraph the figures are shortly described.

Figure (Fig.) 1 is a schematic illustration of the biotin synthesis pathway of *B. sphaericus*.

Fig. 2 is a schematic illustration of the organization of the biotin genes in *E. coli*, *B. sphaericus*, and *B. subtilis*.

Fig. 3 is a schematic illustration of phylogenetic incoherency including the genus *Bacillus*.

Fig. 4 is an illustration of the nucleotide sequence of the *B. subtilis bio* promoter region (SEQ ID NO:7), including amino acid translations of the end of the ORF4-5 reading frame (SEQ ID NO:16) and the beginning of the *bioW* reading frame (SEQ ID NO:17).

Fig. 5A is a physical map of the *B. subtilis biotin* operon.

Fig. 5B is a map showing transcription of the *B. subtilis biotin* operon.

Fig. 6 shows the restriction enzyme sites and complementation results for pBIO plasmids.

Fig. 7 shows the complementation results with deletions and subclones of pBIO201.

Fig. 8 is a physical map of the *B. subtilis bio* promoter region.

Fig. 9 shows the location and Bio phenotype of *cat* (chloramphenicol-acetyl transferase) insertional mutations within *B. subtilis bioW*, ORF2, and ORF3.

Fig. 10 shows the location and Bio phenotype of *cat* insertional mutations within *B. subtilis bio* promoter region, ORF4-5, and ORF6.

Fig. 11 is a comparison of the nucleotide sequences of the *B. sphaericus bioDAYB* regulatory region (SEQ ID NO:8) and the *B. subtilis bio* promoter region (SEQ ID NO:9).

Fig. 12 is an illustration of the restriction sites introduced for the 5'-biotin operon cassette: 1. upstream homology, terminator; 2. Promoter, operator, leader; 3. Ribosome-binding site, start codon, 5'-*bioW*.

Fig. 13A shows the orientation and sequence of the following PCR primers for the 5'-*bio* cassette: ORF4 1' (SEQ ID NO:10), BIOL5' (SEQ ID NO:11), Leader1 (SEQ ID NO:12), ANEB1224 (SEQ ID NO:13), BIOL3 (SEQ ID NO:14), and BIOL4 (SEQ ID NO:15).

Fig. 13B is diagram of the construction of pBIO144.

Fig. 14 shows the DNA sequence of the *B. subtilis biotin* operon and its flanking sequences (SEQ ID NO 1).

Fig. 15 shows the vitamin spectrum of various fermentation broths.

Fig. 16 is an illustration of an in-frame deletion in *bioW*.

Fig. 17A is an illustration of elements of the *bioW* catabolite repression sequence.

Fig. 17B is an illustration of the terminator region deleted between *bioB* and *bioI*.

Fig. 18 is a graph showing the azelaic acid resistance of strains PA3 and PA6.

Fig. 19 is a graph showing the azelaic acid resistance of strains BI535 and BI544.

It is highly desirable to develop an efficient system for producing high titres of biotin that can be economically used in a commercial process. The present applicants have recognized that an improved system for biotin production can be developed using *B. subtilis*.

B. subtilis has several advantages over the use of other species. Unlike *B. sphaericus*, *B. subtilis* is highly characterized for the purposes of genetic engineering, increasing the ease by which one can a) develop a mutant strain optimized for biotin production; and b) manipulate and construct genetic vectors carrying genes encoding the biotin biosynthetic enzymes. Most importantly, applicants disclose herein that most or all of the genes encoding the biotin biosynthetic enzymes are found on a single operon. This makes it easier to generally regulate the expression of the operon, and to co-regulate the amount of each enzyme expressed. Furthermore, *B. subtilis* contains a unique cytochrome-450-like enzyme that is involved in vitamin production and can be manipulated to significantly enhance vitamin production. Neither the gene (*bioI*) encoding this enzyme nor any homologue of it from other organisms has been reported to play a role in biotin or biotin vitamin synthesis.

Obtaining the genes for the *B. subtilis* biotin operon was not a straightforward task. Preliminary attempts to identify the genes based on sequence similarity to *B. sphaericus* failed. The reason for the failure is that, despite their common taxonomic grouping as *Bacillus*, *B. subtilis* and *B. sphaericus* are quite divergent species (Stackebrand et al. *supra*). Consequently the sequence homology between the relevant *B. sphaericus* genes and corresponding *B. subtilis* genes was too low to permit cloning of the *B. subtilis* genes using the *B. sphaericus* genes as probes.

Applicants have therefore used an alternative and more successful strategy to clone the genes required for biotin biosynthesis (*bio* genes). This approach included complementation experiments with *E. coli* mutants in *bioA*, *bioB*, *bioC*, *bioD*, *bioF*, and *bioH*, and further characterization by marker-rescue and complementation experiments with known *B. subtilis* biotin mutants in *bioA*, *bioB*, and *bioF* (Pai et al. *supra*). These experiments showed that in *B. subtilis* all six of these biotin biosynthetic genes are contained on a single DNA fragment of approximately 8 kb. A detailed restriction map of this fragment has been obtained, and an analysis of overlapping clones, deletion mutants, subclones, and their respective nucleotide sequences allowed the genes to be located on the DNA fragment in the order, from right to left, *bioW*, *bioA*, *bioF*, *bioD*, *bioB*, *bioI*, and ORF2. All seven genes are transcribed in the same direction, compatible with their being part of a single operon.

The isolated biotin operon of *B. subtilis* was then inserted into a microbial host for the production of biotin. The operon and the microbial host can each be, or can separately be, deregulated for biotin production in order to provide a maximal level of biotin production.

EXAMPLE I. Cloning the *B. subtilis* genes for biotin biosynthesis.

Applicants have cloned and characterized *B. subtilis* genes required for biotin biosynthesis (*bio* genes). Since prior to this work all that was known concerning *B. subtilis* *bio* genes was that mutations in *bioA*, *bioB* and *bioF* existed and were closely linked on the chromosome (Pai et al., *supra*), two different approaches were originally taken to clone these genes. The first approach involved testing of short (~45-60 bps) probes designed according to conserved sequences, and larger probes (~1 kb) generated by the polymerase chain reaction (PCR) from *B. sphaericus* *bio* genes. However, these probes failed to hybridize specifically to chromosomal digests of *B. subtilis* DNA. A second approach involved screening libraries of *B. subtilis* DNA for recombinant clones that complement *E. coli* *bio* mutants.

IA. Attempts to clone the *bio* genes by DNA hybridization with *B. sphaericus* sequences.

To identify restriction fragments of *B. subtilis* that contained *bio* genes, short (~45-61 bps) probes to internal regions of the *bioA*, *bioB*, and *bioF* genes of *B. sphaericus* were prepared. The sequences of the probes were chosen based on conserved amino acids predicted from the *bio* DNA nucleotide sequences of *E. coli* and *B. sphaericus*.

The characteristics of these probes were as follows: *bioA*, 60-mer and *bioB*, 48-mer (nucleotides #1950-2009 and #3333-3380, respectively, from *B. sphaericus* sequence, GenBank™ accession #M29292). *bioF*, 45-mer (nucleotides #2877-2921 from *B. sphaericus* sequence, GenBank™ accession #M29291). Two of the probes did not hybridize (*bioB* probe) or hybridized poorly (*bioF* probes) to various chromosomal digests of *B. subtilis* DNA even when the stringency of the hybridization conditions was low (5× SSC, 10% formamide, 1× Denhardt's solution, 100 µg/ml single-stranded salmon sperm DNA, 37°C). Only the *bioA* probe was able to hybridize. However, purified DNA fragments identified by *bioA* hybridization failed to marker-rescue the *B. subtilis* *bioA* mutant indicating that the fragments did not contain the *bioA* gene. Furthermore, the DNA hybrids were unstable; the probe could be washed off the filters under conditions of moderate stringency (0.25-0.1 × SSC 37°C). Similarly, larger DNA probes (~1kb) of the three *bio* genes, which were prepared by PCR amplification of *B. sphaericus* chromosomal DNA, also failed to

hybridize specifically with *B. subtilis* chromosomal DNA. Consequently these probes could not be used to screen gene banks for recombinant clones containing *B. subtilis* *bio* genes.

1B. Cloning the *bio* genes by complementation of *E. coli* mutants

5 A library of random *B. subtilis* fragments (~10kb) was constructed in an *E. coli* vector using the positive selection vector pTR264 [Lauer et al., *J. Bact.* 173, 5047-5053 (1991)]. pTR264 is a pBR322 derived vector carrying an ampicillin resistance gene and the λ repressor gene as well as the gene for tetracycline resistance under the control of the regulatory sequences subject to regulation by the λ repressor. pTR264
10 was constructed by reconstituting the ampicillin resistance gene of pTR262 by adding back the 5' end of the gene from the *Pst*I site. A unique *Bcl*I site is located within the λ repressor gene. Insertion of DNA fragments into this site disrupts repressor function, thereby relieving repression of the *tet* gene. Clones with inserts are selected by plating transformants on tetracycline plates.

pTR264, isolated from a *dam*⁻ *E. coli* strain and digested with *Bcl*I, was ligated with *B. subtilis*
15 chromosomal DNA which had been partially digested with *Sau*3A and fractionated on a sucrose gradient (8-12 Kb fragments). The library (labelled BSB1) contained Tet^r plasmids that complemented all the known *E. coli* *bio* point mutations. *E. coli* biotin mutants R879 (*bioA*24), R875 (*bioB*17), R878 (*bioC*23), R877 (*bioD*19), R872 (*bioF*3), and BM7086 (Δ *malA-bioH*) [Cleary and Campbell, *J. Bact.* 112, 830-839 (1972); Hatfield et al., *J. Bact.* 98, 559-567 (1969)] were transformed with the BSB1 library. Plasmids were isolated
20 from each Bio⁺ transformant. Plasmids pBIO100 and pBIO101 were isolated by complementation of R879 (*bioA*); plasmids pBIO102 and pBIO103 by complementation of R877 (*bioD*); plasmid pBIO104 by complementation of R872 (*bioF*); plasmids pBIO109 and pBIO110 by complementation of BM7086 (*bioH*); and plasmids pBIO111 and pBIO112 by complementation of R878 (*bioC*) (Fig. 6). Finally, DNA from the BSB1 library was transformed into *E. coli* BM4062 [*birA*(ts)] [Barker and Campbell, *J. Med. Biol.* 146, 469-492 (1981)]. Plasmids pBIO113 and pBIO114 were isolated from colonies that grew at 42°C.

Initial restriction analysis of the isolated plasmids indicated significant overlap of the cloned DNA fragments, suggesting that the five genes *bioA*, *bioC*, *bioD*, *bioF* and *bioH* are clustered in *B. subtilis* in a single operon. However, the *birA* complementing plasmids pBIO113 and pBIO114 did not overlap with the other five fragments.

1C: Restriction mapping of the *bio* plasmids

A restriction map of the *bio* locus from unique *Eco*RI to *Bam*HI sites is shown in Fig. 6. The *Eco*RI to
35 *Bam*HI fragment cloned into a derivative of pBR322 was called pBIO201. The detailed restriction map of the 9.9 kb fragment in pBIO201 was obtained by standard single and double enzyme digestion analysis.

pBIO100, the first clone of *bio* genes isolated by complementation in *E. coli*, extended an additional 300 bp beyond the *Bam*HI site at one end. pBIO110, isolated by complementation of *bioH* mutants of *E. coli*, extended about 1100 base pairs beyond the *Eco*RI site at the other end. Southern hybridization studies indicated that the insert DNA of pBIO100 was derived from a single continuous segment of the *B. subtilis* chromosome.
40

1D: Complementation/marker rescue of *B. subtilis* and *E. coli* *bio* mutants with pBIO plasmids.

To confirm that the cloned DNA of pBIO100 contained *B. subtilis* *bio* genes, pBIO100 was tested for
45 the ability to marker-rescue *B. subtilis* *bio* mutants. The plasmid restored biotin prototrophy to *bioA*, *bioB*, and *bioF* mutants at high frequency, indicating that the cloned DNA contained all or part of each of these *B. subtilis* *bio* genes.

The pBIO plasmids were also examined for their ability to complement *E. coli* *bio* mutants *bioA*, *bioD*, *bioF*, *bioC* and *bioH*. Most plasmids complemented more than one *E. coli* biotin mutation. The isolate
50 pBIO112 complemented *E. coli* mutations in *bioA*, *bioB*, *bioC*, *bioD*, *bioF* and *bioH* (Fig. 6). pBIO112 did not complement the *E. coli* *birA*(ts) or the Δ (*gal*-*uvrB*) mutation. These data demonstrated that most of the known biotin genes are in a single cluster in *B. subtilis*.

Several deletions in pBIO201 were constructed by cutting at two mapped restriction sites, filling in overhangs if necessary, and ligating. After the structure of the deleted plasmids was confirmed, each was
55 transformed into various *E. coli* *bio* mutants and complementation was scored. The results are summarized in Fig. 7. The deletion derivative pBIO203 was found to complement six of the known *E. coli* biotin genes (*bioA*, *bioB*, *bioC*, *bioD*, *bioF*, *bioH*), establishing that all six of the genes were located in the 8 kb fragment of DNA from *Bam*HI to *Xho*I. The removal of 3.8 kb from the left of this fragment (pBIO204) eliminated the

ability to complement *bioC* and *bioH* mutants. pBIO206 contained only the right most 2.5 kb of the biotin cluster and complemented only *bioA* and *bioF* mutants. The order based on these observations and hybridization data was *bioC*, *bioH*, (*bioB*, *bioD*), *bioF*, *bioA*.

IE: Cloning of a *B. subtilis* fragment containing a complete *bioW*.

DNA sequencing (see below) revealed that the promoter of the *bio* operon was not present on any of the originally cloned DNA fragments. However, this promoter region was recovered by chromosomal walking. None of the clones originally isolated by complementation of *E. coli* mutants had extended further to the right than had pBIO100. This was surprising since *bioA* sat at the rightmost end of these clones and fragments in the 8-10 kb range had been selected for cloning. However, DNA sequences of the rightmost end of the cloned insert of pBIO100 revealed about 300bp of an open reading frame that was somewhat similar to *B. sphaericus bioW*. Fragments containing *bioA* and the adjacent upstream region were cloned in *E. coli bioA* cells containing a *pcnB* mutation to reduce plasmid copy number [Lopilato et al., *Mol. Gen. Genet.* 205, 285-290 (1986)]. Under such conditions a *Pst*I fragment containing an additional 2.7 kb of DNA upstream of *bioA* was cloned, and the location of the beginning of the *bio* operon was determined by DNA sequencing.

The *pcnB* mutation in *E. coli* results in low copy number maintenance of ColE1-derived plasmids, including pBR322 and pUC derivatives [Lopilato et al., 1986, *supra*]. An *E. coli* strain, BI259, that contained both the *bioA* and *pcnB* mutations, was constructed. Restriction enzyme, deletion, and Southern analyses had shown that a 5.5 kb *Pst*I fragment would contain a complete *bioA* gene. *B. subtilis* GP275 chromosomal DNA was digested with *Pst*I and size fractionated by agarose gel electrophoresis. A pool of 4.4 to 6.6 kb fragments was ligated into a pBR322-derived plasmid and used to transform BI259. Selection was for ampicillin resistance and biotin prototrophy. A plasmid, pBIO116, was recovered that contained a 5.5 kb insert. This plasmid could transform BI259 to biotin prototrophy at high frequency but could not transform R879 (*bioA*, *pcnB*⁺) to either biotin prototrophy or ampicillin resistance. Southern hybridization with a probe (a 600 bp *Pst*I-*Bam*HI fragment of pBIO100) containing the 300 bp that was somewhat similar to *B. sphaericus bioW* was used to confirm that the cloned DNA contained the *bioW* homolog.

pBIO116 was available in very limited quantities from the *pcnB* background. The *pcnB80* allele which was used in this cloning experiment is reported to reduce the copy-number of pBR322 replicons to about 6% of wild-type level [Lopilato et al., 1986, *supra*]. To improve plasmid yields without impairing plasmid stability, the DNA was cloned in a low copy-number plasmid. The unique *Bam*HI site within the 3' end of *bioW* was used to subclone a 3.0 kb *Bam*HI-*Pst*I fragment from pBIO116 into pCL1921. pCL1921 is a derivative of the low-copy number plasmid pSC101 that contains the *lacZ'*/polylinker cloning region of pUC19 and a selectable spectinomycin/streptomycin resistance gene [Lerner and Inouye, *Nuc. Acids. Res.* 18:4631, 1990]. pCL1921 has a copy number of about 5-10 copies per cell. Purified 3.0 kb *Bam*HI-*Pst*I DNA from pBIO116 was ligated to *Bam*HI and *Pst*I-cut pCL1921 DNA and the ligated DNA was transformed into a *pcnB*⁻ *E. coli* strain, MM294, selecting for spectinomycin-resistance (100 µg/ml). A plasmid, pBIO350, was recovered that contained the correct 3.0 kb *Bam*HI-*Pst*I fragment. The quantity of pBIO350 recovered from this strain was significantly higher, without loss of plasmid stability, compared to pBIO116 isolated from the *pcnB80* strain.

EXAMPLE II. DNA sequencing of the *B. subtilis bio* gene cluster.

To further identify the *bio* biosynthetic genes, to understand the regulatory apparatus controlling their expression, and to locate sites appropriate for genetic engineering, the *B. subtilis bio* genes contained on clones pBIO100 and pBIO350 were sequenced using the Sanger dideoxy sequencing method using Sequenase™ kits, version 2.0 (United States Biochemicals, Cleveland, OH, USA) as instructed by the manufacturer.

IIA: DNA sequencing strategy

The strategy used to obtain the DNA sequence of the 8-10 kb region that included the *B. subtilis bio* genes was to divide the region into four plasmid subclones of approximately equal size, and then make nested sets of deletions progressing through each subclone. To generate the nested deletions the "exonuclease III - endonuclease S1" method was used; the reagents were purchased in a kit (Promega, Madison, WI, USA). Nested deletions were made from both ends for three of the subclones and from one end for the fourth. Sequencing both sets of nested deletions for three of the subclones gave the sequence

of both strands of each subclone, which is necessary to obtain a completely accurate sequence. For pBIO350 one strand was determined similarly and the opposite strand was determined by synthesizing sequencing primers at intervals of approximately 150 bp. The junctions between non-overlapping subclones were confirmed by sequencing from synthetic primers using pBIO201 or pBIO100 (or subclones thereof) as a template. The sequences were aligned and compared with the DNASTAR computer program (DNASTAR, Inc., Madison, WI, USA).

II B. Identification and organization of bio-specific coding regions and transcriptional regulatory signals.

Analysis of ~8500 bp of the DNA sequence from pBIO100 and pBIO350 indicated a single *bio* operon containing seven coding regions (Fig. 5A, Fig. 14, and SEQ ID NO:1). Starting at the 5' end of the *bio* operon in pBIO350 and continuing through pBIO100, one finds first a ~100 bp region which contains a putative promoter sequence recognized by the vegetative form (σ^A) of *B. subtilis* RNA polymerase (referred to as P_{bio}) next to a transcription regulator site that is defined by a strong sequence homology to the "operator" sites of the *B. sphaericus* *bio* operons.

The nucleotide sequence of this putative promoter region is shown in Fig. 4. [Fig. 4 symbols are as follows. Dashed lines: regions of dyad symmetry; Bold underline: similarity to the *B. sphaericus* *bio*DAYB regulatory site; σ^A : promoter region recognized by the vegetative form of *B. subtilis* RNA polymerase; RBS: ribosome binding site; *: restriction site blocked by *dam* methylation. Deduced amino acid sequences are shown below the nucleotide sequence.] The sequence of P_{bio} is TTGACA -- 17bp -- TATATT (SEQ ID NO:2) and is in good agreement with the *B. subtilis* σ^A consensus sequence, TTGACA -- 17/18 bp -- TATAAT (SEQ ID NO:3). This region is immediately followed by an ORF (open reading frame) with homology to *bioW* (259 amino acids), followed by ORFs with homology to *bioA* (448 amino acids), *bioF* (389 amino acids), *bioD* (231 amino acids), and *bioB* (335 amino acids). The next two open-reading frames ORF1 (*bioI*; 395 amino acids) and ORF2 (253 amino acids) showed no sequence similarity to any known *bio* gene (Fig. 5A). The positions of the promoter, genes and putative transcription termination sites are summarized in Table 1.

Table 1. Summary of Genes, Promoters, and Regulatory Elements in the *B. subtilis* biotin operon.

(Refer to SEQ ID NO:1 and Fig. 14 for numbering of bases)

Gene or Element	Starting Base	Ending Base	Comments
σ^A promoter	324	352	P_{bio}
Potential Regulatory Site	355	387	homology to <i>B. sphaericus</i> <i>bio</i> operator site
<i>bioW</i>	439	1218	ATG start
<i>bioA</i>	1208	2554	ATG start
<i>bioF</i>	2544	3713	TTG start
<i>bioD</i>	3710	4405	TTG start
<i>bioB</i>	4408	5415	ATG start
<i>bioI</i>	5484	6671	GTG start
<i>orf2</i>	6748	7509	GTG start
<i>orf3</i>	7695	--	GTG start
rho-independent termination sites			upstream from 5' promoter between <i>bioB</i> and <i>bioI</i> At 3' end of <i>bio</i> operon
	249	291	
	5423	5462	
	7501	7543	

The location and orientation of promoters, transcriptional termination sites, and ribosome binding sites, are indicative of a single operon containing the *bio* genes. Each gene is preceded by a strong *Bacillus* ribosome binding site (RBS), with calculated ΔG 's ranging from -11.6 to -20.4 kcal/mol. All genes are

oriented in the same transcriptional direction (right to left). In addition, the 5' ends of *bioA*, *bioF*, *bioD*, and *bioB* overlapped the 3' ends of each preceding gene suggesting that expression of these genes is regulated, in part, by translational coupling. *bioI* and ORF2 are separated from the previous gene by 68 and 67 base pair intercistronic regions, respectively, indicating that they are not translationally coupled. The *bioW* gene appears to be the first gene in the operon as it is preceded by the potential RNA polymerase (σ^A) promoter site and putative operator site discussed above. This promoter region represents the beginning of the *bio* operon, since approximately 50 bp upstream from is a stem-loop structure that resembles a *rho*-independent transcription termination site. This putative termination site represents the end of a separate transcription unit since it is in turn preceded by a coding region with a strong *Bacillus* ribosome binding site (RBS; $\Delta G = -14.8$ kcal/mol) labeled ORF4-5 (299 amino acids), which is oriented in the same direction as the *bio* operon (Fig. 8). Finally, further upstream from ORF4-5, oriented in the opposite direction, there is a strong *Bacillus* RBS ($\Delta G = -17.4$ kcal/mol) followed by the first 266 amino acids of another open reading frame, ORF6; the remainder of ORF6 continues beyond the *PstI* site. The deduced amino acid sequence of ORF6 showed significant similarity to a number of regulatory proteins related to the *lacI* repressor: *E. coli ebvR* (a repressor of a cryptic operon of unknown function), *E. coli purR* (a repressor of the purine nucleotide biosynthetic operon), and *E. coli cytR* (a pleiotropic transcriptional repressor of *deoCABD*, *udp*, and *cdd* encoding catabolizing enzymes and *nupC*, *nupG*, and *tsx* encoding transport and pore-forming proteins). Transcription of ORF4-5 and ORF6 may be coordinated since we detect two overlapping σ^A promoter sequences within the 175 bp gap between the 5' ends of ORF4-5 and ORF6 (TTGTAA --18bp -- TAATAT (SEQ ID NO:4) \rightarrow ORF6; TTGATA -- 17bp -- AAAAGT (SEQ ID NO:5) \rightarrow ORF4-5) and a series of inverted repeats.

ORF2 is the last gene in the *bio* operon based on the presence of a stable stem-loop structure resembling a *rho*-independent transcription termination site immediately at the end of this coding region. A second stem-loop structure with terminator-like features was identified in the intercistronic region between *bioB* and *bioI*. Several secondary structures of the mRNA are possible, with the most favored structure having a ΔG of formation of -11 kcal/mol and the least favored structure a ΔG of -5.6 kcal/mol.

Downstream from the end of the biotin operon is a strong RBS ($\Delta G = -20.0$ kcal/mol) and 260 amino acids of another coding region, ORF3. The remainder of ORF3 continues beyond the *BstXI* site which marks the end of the sequenced region. The deduced amino acid sequence of ORF3 showed significant similarity to a number of *E. coli* membrane-associated transport proteins: glycerol-3-phosphate permease (*ugpE* and *ugpA*); maltose permease (*malG* and *malF*); and molybdenum permease (*chf*). In particular, the partial ORF3 peptide contains a 20 amino acid sequence at the COOH-terminal region found common to all membrane-associated transport proteins. ORF3 is transcribed separately from the *bio* operon using a putative σ^A promoter sequence TAGACA-N₈-TACATT (SEQ ID NO:1; Fig. 14, #7600-7629) 95 bps upstream of ORF3.

The gene-enzyme relationships, the enzyme sizes, and percent homology to the same enzyme from other organisms are summarized in Table 2.

Complementation studies using plasmid subclones that contained either *bioI* or ORF2 alone under the transcriptional control of the *lac* promoter indicate that *bioI* alone is sufficient to complement either a *bioC* or *bioH* mutation of *E. coli*. Copies of *bioI* and ORF2 were generated by PCR. A *HindIII* site was introduced at the 5' end of each gene, a *BamHI* site was introduced at the 3' end of *bioI* and an *Asp718I* site was introduced at the 3' end of ORF2. The PCR generated fragments were each cloned into three plasmids with different copy number; the low copy number plasmid pCL1921, a medium copy number plasmid, pJGP44 (which is derived from pBR322), and the high copy number plasmid, pUC19. In two of these recombinant plasmids expression of *bioI* and ORF2 is under the control of the *lac* promoter (pCR1921 and pUC19).

Plasmids containing *bioI* complemented both *E. coli* BM7086 ($\Delta bioH$) and *E. coli* R878 (*bioC*). Plasmids containing ORF2 did not give normal complementation of either *E. coli* BM7086 or R878. It was clear from these experiments that the product of the *bioI* of *B. subtilis* is able to supply an activity needed for biotin synthesis that can substitute for, or overcome, the activity missing in either *bioC* or *bioH* mutants of *E. coli*.

A plasmid (pBIO403) containing only the *B. subtilis bioW* gene and its promoter cloned into pCL1921 (Lerner and Inouye, 1990, supra), complemented both *E. coli* $\Delta bioH$ and *bioC* mutants, if and only if pimelic acid was added to the medium at about 30 mg/l. This experiment confirmed that *bioW* encodes a pimelyl-CoA synthetase that can bypass *bioH* and *bioC* in *E. coli*.

Table 2. Enzymes, genes, and ribosome binding sites of biotin biosynthesis in *B. subtilis*.

Gene	RBS AG (kcal/mol)	Predicted start codon	Enzyme or Function	Estimated no. of amino acids	Estimated M _r	Estimated percent amino acid identity to self-gene from
biotIn biotIn biosynthetic operon (map position 262*)						
bioW	-11.8	ATG	Pimelyl-CoA synthetase	259	29,633	44
bioA	-15.8	ATG	DAPA aminotransferase	448	50,118	34
bioF	-11.6	TTC	7-MAP synthetase	389	42,567	35
bioD	-18.6	TTC	DTB synthetase	231	25,114	32
bioB	-12.2	ATG	Biotin synthetase	335	36,931	34
bioI	-18.4	GTG	Cytochrome P-450	395	44,838	71
ORF2	-17.6	GTG	β-ketoreductase	253	28,204	30 ^b
ORF3	-20.0	GTG	Unknown membrane-associated transport protein	>258	>28,600	33 ^c
Downstream gene						23 ^d
Upstream genes						25 ^e
ORF4-5	-14.8	ATG	Unknown	299	33,780	23 ^f
ORF6	-17.4	ATG	Unknown regulatory protein	>266	>29,200	29 ^g

a identity to *E. coli* lipA.b identity to *Bacillus megaterium* cytochrome P-450_{BM-1}.c identity to *Saccharopolyspora erythraea* eryF.d identity to *Saccharopolyspora erythraea* eryAII.e identity to *E. coli* malG.f identity to *E. coli* upfE.g identity to *E. coli* ebqR.h identity to *E. coli* purR.i identity to *E. coli* cysR.

No significant similarity was detected between the deduced amino acid sequence of either *B. subtilis* *bioI* or ORF2 and the protein sequences of *E. coli* *bioC* or *bioH* genes or other *bio* genes. Subsequent comparison to the protein database of GenBank™, however, indicated significant similarity of *bioI* to a number of cytochrome P-450 enzymes from *B. megaterium* (BM-1), *S. erythraea* (eryF and eryKI), *S. griseolus* (*suaC* and *subC*), *S. species* strain SA-COO (*choP*), and other organisms. Cytochrome P-450s

are a class of enzymes that include monooxygenases that are known to catalyze hydroxylation of many different kinds of substrates, including fatty acids. Since synthesis of pimelic acid, a precursor to biotin, might involve hydroxylation and/or further oxidation of an unidentified fatty acid, *biol* may be involved in an early step in biotin synthesis. The *bioC* and/or *bioH* genes are functionally equivalent to *biol*, based on the ability of *biol* to complement *bioC* and/or *bioH* mutations. Similar comparative studies revealed weak similarity of ORF2 to the β -ketoreductase domain of polyketide synthase II (*ery AII*), which is involved in an early enzymatic step in erythromycin formation.

EXAMPLE III: *cat* insertional mutagenesis of the *bio* operon and flanking coding regions.

To verify the boundaries of the *bio* operon predicted from the nucleotide sequence and to confirm the role of previously unidentified *bio* genes, a *cat* cassette was used to construct insertions or deletions in *bioW*, *biol*, ORF2, the *bio* promoter region, ORF3, ORF4-5, and ORF6 (located outside the predicted boundaries of the *bio* operon). The *cat* cassette includes a chloramphenicol resistance gene. To make the above-mentioned constructions, plasmid derivatives containing these mutations were first constructed in *E. coli*. The *cat* insertions were then transferred to the *bio* chromosomal locus of *B. subtilis* by DNA transformation using standard procedures. To determine whether the insertions or deletions inactivated biotin synthesis, colonies containing these mutations were assessed for growth on biotin-free medium agar plates with or without the presence of biotin (Bio phenotype).

As diagrammed in the top of Fig. 9 and Fig. 10, the *cat* cassette was inserted by ligation into the coding regions of *bioW* using a *Bam*HI site; into *biol* using a *Sma*I site; into ORF3 using a *Xmn*I site; into ORF6 using an *Eco*RV site; between the pair of *Sst*I sites deleting the 3' end of ORF2; or between the pair of *Bst*BI sites deleting ORF4-5. The *cat* cassette was also used to either disrupt the *bio* promoter region by ligating it into the *Eco*47III site, or used to entirely replace this promoter region by ligating it between the *Hpa*I sites. In each of the ORF2/*Sst*I, ORF4-5/*Bst*BI, and *Eco*47III constructions, the *cat* gene was inserted only in the same direction as the disrupted coding region or promoter region. In all other constructions, two different plasmid derivatives were generated where the *cat* cassette was inserted in either possible orientation. Each of these mutations was then integrated into the *bio* locus by first linearizing the *cat*-containing plasmid by a restriction enzyme cut outside of the *bio* DNA and transforming this cut DNA into a competent prototrophic *B. subtilis* strain, PY79, and selecting for chloramphenicol-resistance (*Cm*^r). The Bio phenotype of each mutant is summarized at the bottom of Fig. 9 and Fig. 10. Insertions within the coding regions located outside of the predicted *bio* operon, ORF3, ORF4-5 and ORF6, generated *Cm*^r prototrophic colonies, indicating that these mutations had no phenotype with respect to biotin production and with respect to auxotrophy. Insertions within the *bio* operon gave complex results that generally supported the nucleotide sequence data. Interruption of the *bio* promoter region with the *cat* gene oriented in the opposite direction relative to the biotin operon, and interruption of *bioW* with the *cat* gene oriented in either direction relative to the *bio* operon, generated an unambiguous Bio⁻ phenotype, confirming the location of these sequences at the 5' end of the *bio* operon/promoter region. However, replacement of the *P*_{bio} promoter region with the *cat* gene inserted in the same transcriptional direction as the biotin operon generated Bio⁺ bacteria at a low frequency (0.1%). Bioassay experiments indicated that bacterial biotin vitamer production was increased in the presence of low concentrations of chloramphenicol, suggesting that transcription of the biotin operon was under the control of the *cat* promoter. The 3' end of the operon could not be definitively identified by this genetic method. Insertions within *biol* resulted in *Cm*^r colonies that were partially deficient in biotin production, i.e., grew poorly on biotin-free medium but grew to wild-type levels in the presence of biotin (33 μ g/ml), whereas the ORF2::*cat* mutation generated Bio⁺ colonies. These results suggested that *biol* is not absolutely required for biotin production, and the ORF2 gene product appeared to be dispensable for wild-type growth in the absence of exogenous biotin. No significant effect on biotin production was detected in a *birA* strain (e.g., BI421; see Example XB) containing the ORF2::*cat* mutation. Nevertheless, it is still possible that ORF2 may be required for overproduction of biotin.

The partial biotin-deficient phenotype generated by the *biol*::*cat* mutation, designated as Bio⁺ ⁻, appeared to be caused by inactivation of *biol* rather than by a polar effect because mutations within the downstream genes ORF2 or ORF3 were Bio⁺. To determine whether the Bio⁺ ⁻ phenotype was genuine and to verify that the *biol* gene product was involved in formation of pimelic acid, the *biol*::*cat* mutation was bypassed by feeding pimelic acid. As summarized in Fig. 9, strains of PY79 containing this mutation in either orientation of the *cat* gene grew to wild-type levels on biotin-free medium containing pimelic acid (33 μ g/ml). These results confirm that the *biol* gene product is involved in early biotin formation and that inactivation of this product only partially disrupts biotin production.

EXAMPLE IV: Analysis of the regulatory mechanism of the biotin operon.

Transcription of the divergent *E. coli* *bio* operon *bioABFCD* is regulated by a classical repressor-operator mechanism, involving a repressor encoded by the *birA* locus [Cronan, *Cell* 58, 427-429 (1989)]. This repressor is a bifunctional molecule carrying the holoenzyme synthetase activity at its COOH-terminal end, an activity which converts biotin into biotinoyl-AMP, an adenylated form of biotin, before transferring it to the apocarboxylase enzyme. Biotinoyl-AMP also functions as the co-repressor, the repressor-biotinoyl-AMP complex blocking transcription by binding to an operator site that overlaps the -10 regions of two divergent promoters.

The 5' promoter and regulatory region of the wildtype *bio* operon was characterized in order to replace it with one of several strong and constitutive *B. subtilis* promoters (see Example VII). The most likely site for initiation of transcription of the *B. subtilis* *bio* operon is a σ^A promoter, P_{bio} , approximately 84 bp upstream from *bioW*, the first gene in the operon (Fig. 4). The actual mRNA start site is either one of the two adenosine nucleotides, 3-4 bp downstream from the end of the "TATATT" box, or the guanosine 7 bp from the "TATATT" box. The RNA leader sequence contains a 33 bp segment with strong sequence homology to the "operator" sites of the *B. sphaericus* *bio* operons. Comparison of the nucleotide sequences of this region to the 5' non-coding region of the *B. sphaericus* *bioDAYB* operon is shown in Fig. 11. [Fig. 11 symbols are as follows. Upper sequence (*B. sphaericus* *bioDAYB* regulatory region) 15 bp regulatory region: double bold underline; region of dyad symmetry: dashed line; start site of transcription determined by primer extension mapping: arrows; ribosome binding site: RBS; The "G" and "T" nucleotides were displaced to facilitate sequence alignment. Lower sequence (*B. subtilis* *bio* promoter region) 13 bp putative regulatory region based on similarity to the *B. sphaericus* regulatory region in the upper sequence: single bold underline, putative start sites of transcription: arrows; ribosome binding site: RBS; The "C" nucleotide was displaced to facilitate sequence alignment.]

The majority of conserved nucleotides are clustered at two sites (13 and 11 bp) separated by a 9 bp segment. This finding suggests that transcription of the *B. subtilis* *bio* genes is regulated by a repressor-operator mechanism, possibly involving a *birA*-like gene located near the *trp* operon (see Example IX). The activity and regulation of a promoter upstream of *bioW* has been verified by showing that a translational *lacZ* fusion to *bioW*, which included P_{bio} and the putative regulatory region, displays biotin-regulated expression of β -galactosidase (see Example V).

The 5' RNA leader contains a potential large stable stem-loop structure ($\Delta G = -14.0$ kcal/mol) that overlaps the operator region.

Based on the identification of P_{bio} and a regulatory site upstream from *bioW*, the *B. subtilis* *bio* genes are transcribed as a single polycistronic message of approximately 7200 bp. In addition, there also exists possible secondary promoter sites located within the internal regions of the *bio* operon. For example, a sequence, TTGAAA -- 17 bp -- TCTTAT (SEQ ID NO:6 and Fig. 14, #6258 to 6286), with some similarity to a consensus σ^A promoter sequence, is located within *bioI* (approximately 775 bp downstream from the start codon of *bioI*). Determination of whether these sequences function as internal promoters can be achieved by using restriction fragments from internal portions of the *bio* operon to construct translational *lacZ* fusions (see Example V). Optimization of biotin production can be achieved by modifying one or more of the primary or secondary promoters.

IVA. Construction and analysis of *bio-lacZ* translational fusions.

A translational *lacZ* fusion was constructed to confirm the activity and regulation of the putative promoter and regulatory region, and to assess the relative level of expression of the *B. subtilis* biotin operon in a variety of contexts. This was accomplished by inserting a 3.1 kb *Bam*HI-*Bgl*II fragment containing a promoter-less *lacZ* coding region into the *Bam*HI site of pBIO350, to give pBIO397 and pBIO398. These two presumably identical plasmids contain an in frame "translational" fusion between *bioW* and *lacZ* on a low copy number plasmid. pBIO397 and pBIO398 turn a *lacZ*⁻ *E. coli* pale blue on X-gal indicator plates, suggesting that the fusion is expressed at a relatively low level in *E. coli*.

To test the *bioW-lacZ* fusion for biotin-regulated expression in *B. subtilis*, Bio⁻ partial diploids were constructed. The *cat* cassette was cloned into pBIO397 using the single *Sma*I site located within the polylinker region downstream from the *lacZ* gene. One recombinant plasmid, pBIO397*cat*, with the *cat* gene oriented in the same direction as the translational fusion, was used to generate Bio⁺ partial diploids. Competent cells of the prototrophic *B. subtilis* strain PY79 were transformed with pBIO397*cat* plasmid DNA selecting for Cm^r. Transformants should only arise by recombination into the chromosome. This leaves an intact copy of the *bio* operon, allowing activity of the *lac* fusion to be assessed in the absence of added

biotin.

The promoter of the *Bam*HI to *Pst*II fragment cloned in pBIO350 is regulated by biotin. Two *Cm*^r *Bio*⁺ partial diploids containing the *bioW-lacZ* fusion were tested for β -galactosidase activity in biotin-free medium in the presence or absence of biotin (100 μ g/liter). In liquid ONPG assays, both strains were regulated specifically by biotin. The level of β -galactosidase was repressed in the presence of biotin and induced in its absence to 24 to 85-fold higher specific activity (Table 3). This strain with the *bioW::lacZ* fusion integrated at the *bio* operon has also been used to isolate new biotin analog-resistant mutants (see below) on X-gal indicator plates.

Table 3

Biotin-regulated expression of <i>bioW-lacZ</i> translational fusion.				
Strain	OD ₅₀₀	β -galactosidase activity (Miller Units) ^a		
		+ Biotin (100 μ g/l)	-Biotin	Fold Increase
PY79(<i>bioW-lacZ</i>)12A	Late Log (0.7-0.8)	0.47	11.30	24
	Stationary (1.1-1.4)	0.17	11.00	65
	Late Stationary (1.4-1.6)	0.13	6.85	53
PY79(<i>bioW-lacZ</i>)14A	Late Log (0.7-0.8)	0.64	21.10	33
	Stationary (1.1-1.4)	0.15	12.30	82
	Late Stationary (1.4-1.6)	0.10	8.45	85
PY79(SP β :: <i>bioW-lacZ</i>)#1 (1.04-1.08)		0.04	0.60	15
P779(SP β :: <i>bioW-lacZ</i>)#3 (0.988-0.960)		0.06	0.49	9
BI421(SP β :: <i>bioW-lacZ</i>)#1 (1.00-0.936)		0.67	1.00	1.5
BI421(SP β :: <i>bioW-lacZ</i>)#3 (1.07-0.92)		0.96	0.86	0.9
α -DB9(SP β :: <i>bioW-lacZ</i>)#1 (0.892-0.528)		0.18	1.65	9.2
α -DB9(SP β :: <i>bioW-lacZ</i>)#3 (1.344-1.200)		0.11	1.35	12.3
HB3(SP β :: <i>bioW-lacZ</i>)#1 (1.068-1.076)		0.24	0.32	1.3
HB3(SP β :: <i>bioW-lacZ</i>)#3 (1.213-1.320)		0.18	0.15	0.8

^a Average of two measurements, except for α -DB9(SP β ::*bioW-lacZ*)#1 (one measurement).

IVB. Construction and analysis of an SP β -borne *bioW-lacZ* translational fusion.

Other strains, in addition to the *lacZ* fusion strain of Example IVA, can be constructed for deciphering biotin regulatory mechanisms. For example, Applicants recognized that insertion of the plasmid-borne *bioW-lacZ* fusion into the chromosome causes technical problems because the integrated plasmid amplifies in copy number. Thus the expression of the *bioW-lacZ* fusion at single copy-number and at a site distinct from the *bio* operon was tested by introducing the fusion into a modified SP β specialized transducing phage [see, e.g., Zuber et al., *J. Bacteriol.* 169, 2223-2230 (1987)].

Two isolates of PY79 (SP β ::*bioW-lacZ*) and BI421 (SP β ::*bioW-lacZ*) were assayed for β -galactosidase activity as described above. The results are summarized in Table 3.

In the *Bio*⁺ strain PY79, expression of SP β ::*bioW-lacZ* was very low, but showed biotin-specific regulation. β -galactosidase was repressed in the presence of biotin and induced approximately 9-15 fold in the absence of biotin. Comparison to earlier assays of PY79 containing two or more copies of the plasmid-borne *bioW-lacZ* fusion indicated that the single-copy fusion produced three-fold less β -galactosidase under repressed growth conditions and about 20-fold less under derepressed growth conditions. In a *B. subtilis birA* strain (BI421; see Example XB) low-level constitutive expression of the fusion was observed. The levels of β -galactosidase in the *birA* strain were only slightly higher than the level observed in PY79 (SP β ::*bioW-lacZ*) grown under derepressed growth conditions. In one of the BI421 (SP β ::*bioW-lacZ*) isolates biotin was slightly repressed β -galactosidase expression, suggesting that this *birA* mutation may not completely relieve biotin regulation of the fusion. These results confirmed that expression of *bioW* is

regulated by biotin

Biotin regulation was also examined in two independently isolated biotin analog-resistant mutants. HB3 contains a spontaneous *birA* mutation and α -DB9 contains an α -dehydrobiotin resistant mutation unlinked to either *bio* or *birA*. These strains were transduced, grown, and assayed as described above, except that α -DB9 (SP β ::*bioW-lacZ*) was assayed during mid-exponential growth. As summarized in Table 3, the fusion-bearing HB3 strains displayed low-level constitutive *lacZ* expression. Unlike the *birA* mutants, however, α -DB9 (SP β ::*bioW-lacZ*) displayed biotin-regulated expression of *lacZ*.

EXAMPLE V: Secretion of biotin and biotin vitamers from *E. coli* strains containing *B. subtilis bio* genes.

During complementation experiments, Applicants observed that an *E. coli bioA* mutant containing pBIO201 could cross-feed the same strain containing the control plasmid pBR322, demonstrating that biotin synthesized by the pBIO201 containing strain was secreted into the media and metabolized by the *Bio*⁻ strain. Applicants therefore tested various newly constructed plasmids for the ability to secrete biotin and biotin vitamers into the media.

E. coli strains MM294 and JM109 *lacI*^Q (both strains are wild-type for *bio* genes) were transformed with pBR322, pBIO201, pUC19, and pBIO289 (described in Example VI, below). The pBR322 and pBIO201 transformants were grown in minimal medium containing 2% glucose. The pUC19 and pBIO289 transformants were grown in a rich medium containing 2% glycerol since they did not grow well in liquid minimal medium. After 48 hours, cells were removed by centrifugation and any residual live cells were killed with chloroform. Supernatants were diluted serially in ten-fold diluted Difco Biotin Assay Medium supplemented with 0.5% glucose and 5 mg/l thiamine, and tested for support of growth of *E. coli* Δ (*mal-bioH*) and *E. coli* Δ (*bioA-D*), described below. Standard curves were prepared from serial dilutions of biotin and desthiobiotin. The assay was sensitive to 1 μ g/liter.

The results from these assays are shown in Table 4. Strains containing plasmids encoding *B. subtilis bio* genes secreted biotin, while strains containing control plasmids did not secrete biotin. This demonstrated that the *E. coli* strains containing the *B. subtilis* biotin operon (pBIO289) are capable of secreting enhanced levels of biotin and biotin precursors.

Table 4

Production of biotin and biotin vitamers* from <i>E. coli</i> strains containing <i>B. subtilis bio</i> genes.			
Strain	Plasmid	Biotin (μ g/l)	Total Biotin and Biotin Vitamers (μ g/l)*
MM294	pBR322	0	3
	pBIO201	10	10
JM109	pUC19	0	0
	pBIO289	10	10
MM294	pUC19	0	1
	pBIO289	10	100

* Biotin vitamers are given as desthiobiotin equivalents.

This assay can also be used to test the level of biotin, or biotin precursor, produced by other strains, e.g., *B. subtilis* strains, or other plasmids. Candidate strains are tested after being transformed with a plasmid bearing a functional biotin operon. Alternatively, candidate plasmids are tested in a strain known to secrete biotin.

EXAMPLE VI: Construction of a "minimal" *bio* subclone.

The minimal subclone encodes all of the relevant functions of the original primary clones (e.g., pBIO100 and pBIO201). A "minimal" subclone was constructed to confirm the location of the *bio* genes derived from deletion mapping and DNA sequence information, and to confirm that the open reading frame downstream from a transcription terminator to the right of an *EcoRV* site (see Fig. 5A) is not required for biotin biosynthesis.

An *EcoRV* (partial) to *BamHI* fragment from pBIO201 (containing all of the open reading frames thought to be *bio* genes except the *bioW* gene) was inserted into the *SmaI* to *BamHI* backbone of pUC9 [Viera and Messing, *Gene* 19, 259-268 (1982)] to construct pBIO289. Since the *bio* genes of pBIO289 are all downstream from the *lac* promoter of pUC9, and since pUC9 is maintained at a higher copy-number than pBR322 (the parent of pBIO100 and pBIO201), pBIO289 is expected to express the *bio* genes at a higher level than pBIO201 and pBIO100 in an *E. coli* host. pBIO100, 201, and 289, as well as pBR322 (as a control), were transformed into a series of isogenic *E. coli bio* mutants. Each transformant was tested for complementation on medium lacking biotin. The results are shown in Table 4. pBIO289 complemented all mutants that were defective in a single *bio* gene, confirming that all relevant genes lie upstream of the putative terminator. The open reading frame downstream from the terminator (ORF3) is not necessary for complementing any of the individual *E. coli bio* mutants.

Table 5

Complementation of various <i>E. coli bio</i> mutants by selected plasmids.				
Mutation	Plasmid			
	pBR322	pBIO100	pBIO201	pBIO289
none	+++	+++	+++	+++
$\Delta(gal-uvrB)$	-	-	-	-
$\Delta bioA$	-	+	++	+++
<i>bioB</i>	-	+++	+++	+++
<i>bioC23</i>	-	+++	+++	+++
$\Delta bioD$	-	-	-	+++
$\Delta(mal-bioH)$	-	+++	+++	+++

EXAMPLE VII. Construction of full length wild-type and engineered *B. subtilis* biotin operons.

Experiments to construct engineered, full length *bio* operons for integration and amplification in the *B. subtilis* chromosome are described below.

VIIA: Re-construction of a full length wild-type *bio* operon

Since it had proved necessary to clone the 5' end of the *B. subtilis* biotin operon at low copy number for work in *E. coli*, a low copy number plasmid was also used for construction of complete and engineered biotin operons for integration into the *B. subtilis* chromosome. As described above, the 5' end of the *B. subtilis bio* operon was cloned as a 3 kb *PstI-BamHI* fragment in pCL1921 (Lerner and Inouye, 1990, supra) a low copy number plasmid present at about 5-10 copies per cell, to give pBIO350.

A full length biotin operon was then reconstructed by adding the 3' portion of the *bio* operon from pBIO201 to pBIO350. A 10 kb *BamHI* to *EcoRI* fragment from pBIO201, that contains the majority of the biotin operon as well as about 3kb of downstream DNA, was ligated into pBIO350 that had been gapped with *BamHI* and *EcoRI*. Two resulting plasmids that had the correct anticipated structure were called pBIO400 and pBIO401. pBIO401 complements all known *E. coli bio* mutants, including a $\Delta bioA-D$ mutant. When pBIO401 was selected by complementation in a $\Delta bioA-D$ strain and then produced in a rich medium, it was stable enough to yield usable quantities of plasmid DNA. Since the spectinomycin-resistance gene carried by pBIO401 is not expressed in *B. subtilis*, a *cat* cassette was added at the *EcoRI* site in pBIO401 to allow for selection, integration, and amplification in *B. subtilis* wild-type or deregulated strains by methods known to those skilled in the art, resulting in the plasmid pBIO401*cat*_s.

To determine the effect of increased *bio* copy-number on biotin production, pBIO401*cat*_s, which contained a single copy of the *cat* cassette (resistant to chloramphenicol at 5 µg/ml) and the entire *bio* operon, was integrated into the chromosomes of wild-type and biotin-deregulated strains of *B. subtilis*. The plasmid copy number was amplified by selection on 60 µg/ml chloramphenicol, and biotin production was thereby increased.

VII B: Construction of engineered bio operons

In constructing engineered, deregulated biotin operons for integration into the *B. subtilis* chromosome, it was useful to install unique restriction sites between transcriptional signals, regulatory sites and coding regions to allow easy introduction of alternate elements or alleles. Also, unique sites were used to flank the engineered biotin operon in these constructions, so as to remove *E. coli*-derived vector sequences prior to integration.

Applicants discovered that construction of an engineered *B. subtilis* bio operons with a strong, constitutive promoter was not a straight-forward task. It was not possible to maintain the entire *B. subtilis* biotin operon on a single plasmid in *E. coli*, even a low-copy plasmid, when the operon was transcribed by a strong constitutive promoter, e.g., the SP01-15 or SP01-26 promoter. An alternative and novel strategy had to be developed to introduce an amplifiable DNA fragment containing the entire engineered bio operon into the *B. subtilis* chromosome. First, for cloning and engineering purposes the operon was manipulated in two separate pieces: 5' and 3' cassettes. Next, when the DNA engineering was completed, the relevant DNA fragments from the appropriate 5' and 3' cassettes were ligated and the ligated cassettes were transformed directly into *B. subtilis*. The ligations were designed to deliver either circular or concatameric molecules that would recombine with homologous sequences in the chromosome thereby inserting the engineered DNA in a manner that could be amplified, with or without accompanying vector sequences.

Plasmids were constructed for use as backbone vectors for developing constructs that include an engineered bio operon. These plasmids were based on the low copy number vector pCL1920 (Lerner and Incuyc, 1990, supra). The polylinker in pCL1920 was replaced with a polylinker flanked by *NotI* sites. The *lac* promoter present in pCL1920 was also removed for simplicity. The fragment containing the *lac* promoter and the polylinker was eliminated with *EcoRI*. The backbone, containing the pSC101 origin of replication and the *omega* fragment encoding resistance to spectinomycin, was re-circularized in the presence of a *NotI* linker to give pBIO121. Plasmid pJGP40 is a pBR322 derivative that contains a kanamycin resistance gene cloned into a polylinker flanked by *NotI* sites. The *NotI* fragment encoding kanamycin resistance from pJGP40 was cloned into the *NotI* site of pBIO121 to give pBIO124, the two orientations being indicated by "a" and "b" (Fig. 12). Digestion of both pBIO124 derivatives with *Asp718I* and religation eliminated the kanamycin resistance element, leaving a complete polylinker, to give plasmids pBIO126a and pBIO126b.

VII Bi: Engineering a 5' Cassette

In considering which functional elements at the 5' end of the bio operon should be separated by unique restriction sites, the elements of most interest were 1) the putative stem-loop termination site upstream from the putative biotin promoter, 2) the putative promoter-operator-leader region, and 3) the ribosome binding site, initiation codon, and 5' coding region of *bioW*. Our strategy, as depicted in Fig. 12, was to introduce by PCR *HindIII* and *SalI* sites 70 bp upstream from the terminator, which is upstream from the biotin promoter. To separate the terminator from the promoter-operator region the *ClaI* site in this region was converted to a *XhoI* site. Conversion of the *Eco47III* site, which precedes the ribosome binding site of *bioW*, to an *XbaI* site separates the promoter-operator-leader region from the ribosome binding site-start codon fragment. A description of all the PCR primers used and their orientation is indicated in Fig. 13A. Table 6 lists the fragments generated by PCR. A three way ligation with 1) PCR fragment E which introduces a 5' *HindIII*-*SalI* site and converts the *Eco47III* site to a *XbaI* site, 2) PCR fragment B which converts the *Eco47III* site to a *XbaI* site and extends to the *BamHI* site in *bioW* and 3) a *HindIII*-*BamHI* digest of pBIO126A vector, resulted in plasmid pBIO139. A second three way ligation with PCR fragments C, D, and *SalI* and *XbaI* digested pBIO139 converted the *ClaI* site to a *XhoI* site and completed the initial construction as plasmid pBIO144. pBIO144 contains a modified wild-type 5' end of the bio operon with a unique *XhoI* site replacing the *ClaI* site upstream of the promoter/operator region and a unique *XbaI* site replacing the *Eco47III* site immediately downstream of this region (Fig. 13A). The expected DNA sequence of pBIO144 from the *SalI* site to the *BamHI* site was confirmed.

VII Bii: Engineered 3' bio cassettes

3'cassettes were constructed in pBIO126A, a low copy number plasmid with a *NotI*-flanked polylinker. To enable pBIO126A to be used as a vector for integration and amplification, a PCR fragment of the *cat* gene from pHW9 [Hornouchi and Weisblum, *J. Bacteriol.*, 150, 815-825 (1982)] was introduced at the *BstBI* site to give plasmid pBIO146A. The *BamHI* to *EcoRI* fragments from pBIO201 (Example IC) and pBIO289

(Example VI) were cloned into the polylinker of pBIO146A to give plasmids pBIO151 and pBIO152 respectively. The two plasmids vary in the amount of 3' flanking sequence accompanying the *bio* operon.

VIIIC. Regulation of engineered *bio* operons by a constitutive promoter.

Different constitutive promoters, such as those from the SP01 bacteriophage, e.g., SP01-26 or SP01-15 [Lee and Pero, *J. Mol. Biol.* 152, 247-265 (1981)], can be added in place of the *bio* promoter/operator region between the *Xho*I and *Xba*I sites. After being integrated and amplified in the *B. subtilis* chromosome, this results in a vector capable of directing expression of the entire biotin operon from a constitutive promoter. This in turn leads to substantially improved biotin production.

Table 6

PCR generated fragments for 5' <i>bio</i> cassette construction				
PCR Fragment	Upstream primer	Downstream primer	bp*	Functional Elements
B	Lcadcr1	ANEB1224	733	RBS, start codon, 5' <i>bioW</i>
C	ORF4.1	BIOL3	140	Upstream homology, terminator
D	BIOL4	BIOL5	95	Promoter, operator, leader
E	ORF4.1	BIOL5	235	Upstream homology, terminator promoter, operator, leader

* fragment size in bp after digestion with restriction endonucleases.

VIIICi. Construction of a 5' cassette with the SP01-26 promoter.

A 5' cassette with the SP01-26 promoter reading into the biotin operon was constructed by replacing the *Xho*I to *Xba*I fragment from the engineered "wild-type" promoter with a PCR fragment containing the SP01-26 promoter. The PCR fragment containing the SP01-26 promoter was generated from pNH201, a pUC8 subclone of the cloned SP01-26 promoter [Lee, G., Talkington, C. and Pero, J. *Mol. Gen. Genet.* 180, 57-65 (1980)]. The primers used (XHO26A and XBA26B, Table 6A) introduced an *Xho*I site at the upstream side of the promoter and an *Xba*I site at the downstream side of the promoter. The *Xho*I-*Xba*I digested PCR fragment was ligated with *Xho*I-*Xba*I digested pBIO144, and the ligated DNA was transformed into *E. coli* YMC9. Plasmid minipreps from Spec^r transformants were screened for acquisition of an *Eco*RV site that is located within the SP01-26 promoter region. Plasmids showing the expected *Eco*RV site were then screened by PCR, using primers XHO26A and BLOW1, to confirm the juxtaposition of the SP01-26 promoter and 5' *bioW* fragments. Two plasmids with the correct structure, pBIO158 and pBIO159, each had the expected sequence.

VIIICii. Construction of a 5' cassette with the SP01-15 promoter.

A DNA fragment containing the SP01-15 promoter (Lee et al., supra) with appropriate ends for cloning in pBIO144 was also generated by PCR. The primers (XHO15B and XBA15C, Table 6A) were selected to generate a fragment which flanked the SP01-15 promoter with *Xho*I (upstream) and *Xba*I (downstream) sites. There is also a potential stem-loop structure near the beginning of the transcript from SP01-15. The downstream primer was also designed to extend the potential stem-loop at the new 5' end of the *bio* mRNA to include the expected +1 base of the transcript initiating by the SP01-15 promoter. The *Xho*I to *Xba*I fragment containing SP01-15 was ligated into *Xho*I-*Xba*I digested pBIO144. This ligated DNA was transformed into *E. coli* to make pBIO168 and pBIO169. pBIO168 and pBIO169 are identical isolates that contain a 5' *bio* cassette with the SP01-15 promoter.

Table 6A. PCR primers

Name	DNA Sequence of Primer
XHO26A	5'GGCCCTCGAG GCCTACCTAG CTTCCAAGAA3'
XBA26B	5'GGCCTCTAGA GCGTCCTGCT GTTGTTAAGA3'
BIOW1	5'GCCAATCCAT TCTGGAGA3'
XHO15B	5'GGCCCTCGAG GCTATTGACG ACAGCTATGG TT3'
XBA15C	5'GGCCTCTAGA ACAGGCGGGG TTGCCCCCGC CTGTAATTAA ATTATTACAC A3'

VIIIciii: Construction and integration of full length engineered "wild-type" bio operons.

Full length engineered biotin operons were constructed by introducing *Sal*I to *Bam*HI fragments from various engineered 5' *bio* cassettes, described above, between the *Sal*I and *Bam*HI sites of pBIO151 and pBIO152, which contain the 3' end of the *bio* operon and a selectable *cat* gene (Example VIII (b)(iii), above). Appropriate transformants were selected by complementation of the *E. coli* strain RY607 (Δbio) to Bio⁺. For example, introduction of the *Sal*I to *Bam*HI fragment from the "wild-type" engineered 5' biotin operon of pBIO144 into pBIO151 resulted in pBIO155 containing the full length wild-type *bio* operon with the 3' flanking region, and a *cat* gene oriented in the same direction as the biotin operon. Ligation of the same 5' fragment from pBIO144 into pBIO152 gave pBIO156 with the same features as pBIO155 but lacking the 3' flanking region downstream of the *bio* operon.

Plasmids pBIO155 and pBIO156 were integrated into *B. subtilis* with the entire plasmid or with the *E. coli* vector sequences deleted (Table 7). *B. subtilis* strains PY79, BI421 and HB3 (both *birA* mutants; see Example X) were transformed with plasmids pBIO155 and pBIO156 and chloramphenicol resistant colonies were selected. Amplification of the integrated plasmid was achieved by streaking the strains on plates with increasing levels of chloramphenicol (Table 7, strains BI228, 230, 235, 237, 243 and 245).

To construct strains which contained amplified copies of the wild-type biotin operon but no *E. coli* sequences, plasmids pBIO155 and pBIO156 were digested with *Not*I. The larger fragment from each digest was circularized and used to transform *B. subtilis* strains PY79, BI421, and HB3. The cassette was amplified by streaking on plates with increasing levels of chloramphenicol (Table 7, strains BI232, 234, 239, 241, 247, and 249).

SP01 promoter driven *bio* operon ligations were transformed directly into *B. subtilis*. Isolated *Not*I to *Bam*HI fragments from 5' cassettes (5' flanking sequence, promoter region and 5' *bioW*) and isolated *Bam*HI to *Not*I fragments from 3' cassettes (3' *bioW*, *bioA*, *bioF*, *bioD*, *bioB*, *bioI*, ORF2, terminator, 3' flanking sequence, and *cat*) were ligated under standard conditions and used to transform various *B. subtilis* strains. The 5' *Not*I to *Bam*HI cassettes were from pBIO158 containing the SP01-26 promoter or pBIO168 with the SP01-15 promoter. The 3' cassettes were from either pBIO151 with the extended 3' flanking sequence or pBIO152 with the truncated 3' flanking sequence (Table 7, strains BI267, 268, 274, 276, 278, and 282).

Each of these DNA ligations was transformed into a wild-type strain PY79 and in some cases also into a biotin-deregulated strain, BI421 (*birA* mutant, see Example XB below). Competent cells of the strains were prepared by standard methods and transformed with the different *bio*-containing DNA ligation mixtures described above, selecting for Cm^r. Since these DNA's cannot replicate in *B. subtilis*, Cm^r transformants arise by integration of the ligated DNA into the chromosome at the *bio* locus via recombination between homologous sequences present on the chromosome and the transforming DNA. In each experiment, 10-50 Cm^r transformants were selected for characterization. Transformants were screened by PCR to confirm that the SP01 promoter was juxtaposed to *bioW*.

Table 7

First generation biotin production strains.					
Strain Sequences	Parent	Integrated DNA	Promoter	3' Flanking	
BI222	PY79	pBIO401cat _s	biotin	yes	
BI224	BI421	pBIO401cat _s	biotin	yes	
BI226	HB3	pBIO401cat _s	biotin	yes	
BI228	PY79	pBIO155	biotin	yes	
BI230	PY79	pBIO156	biotin	no	
BI232	PY79	NotI fragment pBIO155	biotin	yes	
BI234	PY79	NotI fragment pBIO156	biotin	no	
BI235	BI421	pBIO155	biotin	yes	
BI237	BI421	pBIO156	biotin	no	
BI239	BI421	NotI fragment pBIO155	biotin	yes	
BI241	BI421	NotI fragment pBIO156	biotin	no	
BI243	HB3	pBIO155	biotin	yes	
BI245	HB3	pBIO156	biotin	no	
BI247	HB3	NotI fragment pBIO155	biotin	yes	
BI249	HB3	NotI fragment pBIO156	biotin	no	
BI267	PY79	5'pBIO158, 3'pBIO151	SP01-26	yes	
BI268	BI421	5'pBIO158, 3'pBIO151	SP01-26	yes	
BI274	PY79	5'pBIO158, 3'pBIO152	SP01-26	no	
BI276	BI421	5'pBIO158, 3'pBIO152	SP01-26	no	
BI278	PY79	5'pBIO168, 3'pBIO151	SP01-15	yes	
BI282	PY79	5'pBIO168, 3'pBIO152	SP01-15	no	

EXAMPLE VIII. Characterization of first generation *B. subtilis* biotin production strains.

Southern blot experiments were used to confirm the structure of the integrated cassettes and to assess the degree of amplification of a representative subset of the engineered strains. From these experiments, it was clear that the presence of the SP01 promoters had a significant effect on the degree of amplification. Engineered, full-length *bio* operons containing a wild-type *bio* promoter were amplified in strains grown on 60 µg/ml chloramphenicol to levels similar to those seen for other operons under similar conditions (estimated 15 copies/cell). However, *bio* operons driven from an SP01-15 promoter showed 2-fold less amplification and *bio* operons driven from the SP01-26 promoter were about four-fold less amplified. Thus, *B. subtilis* cells have a limited tolerance for at least one of the products encoded by the *bio* operon.

IIA. Assay for production of biotin and biotin precursors in test tube cultures.

To determine the effect of multiple copies of the wild-type *bio* operon or SP01-modified *bio* operons on biotin production, the wild-type and biotin-deregulated *B. subtilis* strains containing these engineered *bio* operons, integrated and amplified in their chromosomes, were tested for biotin production. The results are shown in Table 8.

All of the strains were grown overnight in 5 ml of VY medium at 37°C, centrifuged, and the supernatant solutions autoclaved for 5 minutes to kill any remaining cells. (Biotin and desthiobiotin are stable to autoclaving.) The supernatant solutions were diluted in biotin-free medium and inoculated with *E. coli* strains RY604 ($\Delta bioH$) and RY607 ($\Delta bioABFCD$). RY604 and RY607 were constructed by transducing the relevant regions from BM7086 and a $\Delta(gal-uvrB219)$ strain, respectively, (Cleary and Campbell, supra; Hatfield et al., supra) into MM294. The former grows on both biotin and biotin vitamers, while the latter grows on biotin only. The biotin and biotin vitamers produced by different *B. subtilis* mutant strains were calculated from a standard curve at OD₆₀₀.

The wild-type strain of *B. subtilis*, PY79, typically yielded about 6-10 µg/l of biotin in this assay (Table 8). The VY medium used for these experiments had 20-45 µg/l biotin before cell growth. Thus, most of the biotin contributed by the medium was consumed during growth by wild-type bacteria.

Several biotin analog-resistant mutants produced 50-100 $\mu\text{g/l}$ biotin, 5-10 fold more biotin than found with the wild-type strain. Two biotin analog-resistant strains with *birA*-like mutations were used. One mutant strain, HB3, contains a spontaneous homobiotin-resistant mutation. The other strain, BI421, contains an ethylmethylsulfate-generated α -dehydrobiotin-resistant mutation which had been crossed into an un-

mutagenized background (see Example X). Both strains yielded 50-100 $\mu\text{g/l}$ of biotin in these experiments (Tables 8 and 9).

Integration and amplification of a "wild-type" copy of the *bio* operon in the wild-type strain PY79 generally improved biotin production 10-50 fold over that seen with PY79 alone (Table 8). Such strains (BI230 and BI234) produced 150-600 $\mu\text{g/l}$ compared to the 6-10 $\mu\text{g/l}$ produced by PY79 alone. However, more dramatic results were seen from the assays of the *birA* mutant strains with integrated and amplified copies of the wild-type *bio* operon. An additional 5-10 fold improvement in biotin production was observed with yields up to 2,000 $\mu\text{g/l}$ biotin, with this assay (see BI241, BI237, BI249; Table 8).

Analysis of wild-type *B. subtilis* strains containing the engineered *bio* operons with an SP01 promoter resulted in an improvement in biotin production, with biotin titers generally 1000-2000 $\mu\text{g/l}$ (see BI267 and BI274; Table 9) versus 150-600 $\mu\text{g/l}$ with the wild-type *bio* promoter. No dramatic difference was seen in biotin production between wild-type and *birA* mutant strains containing constitutive promoters (Table 9).

A second type of assay employs *Lactobacillus plantarum* as a biotin indicator (Wright et al., *Proc. Soc. Exp. Biol. Med.* 56:95-98, 1944) and *Saccharomyces cerevisiae* as an indicator of biotin vitamers (Baldet et al., *Eur. J. Biochem.* 217:479-485, 1993). Assays were performed as described except that an antibiotic was added to the assay cultures to reduce interference by contamination. Since *L. plantarum* is sensitive to most antibiotics, a spontaneous streptomycin resistant mutant, *L. plantarum* str3, was selected and used for biotin assays in the presence of 50 $\mu\text{g/ml}$ streptomycin sulfate. *S. cerevisiae* is naturally resistant to most antibacterial compounds and was also used in the presence of 50 $\mu\text{g/ml}$ streptomycin sulfate. The *L. plantarum* growth response to biotin decreases more gradually (over a dilution range of about 50-fold) than the *E. coli* growth response. *S. cerevisiae* is more responsive to DAPA and KAPA than *E. coli*.

When cultures were assayed for biotin production with *Lactobacillus* as an indicator, more precise levels could be determined. Using these conditions, *B. subtilis* strains with the engineered SP01-*bio* operons yielded almost twice as much biotin as deregulated *B. subtilis* strains with amplified copies of the wild-type *bio* operon (Table 10).

Table 8

Biotin production by various <i>B. subtilis</i> strains containing integrated and amplified wild-type <i>bio</i> operons in test tube cultures.			
Strain name	Relevant features	Biotin ($\mu\text{g/l}$)*	Biotin & Biotin Vitamers ($\mu\text{g/l}$)**
PY79A	prototroph	10	10
PY79B	prototroph	6	6
HB3	<i>birA</i>	100	100
BI230	PY79::[pBIO156] ₀	150	250
BI234	PY79::[Not/156] ₀	600	1,200
BI245	HB3::[pBIO156] ₀	500	2,000
BI249	HB3::[Not/156] ₀	1,500	1,500
BI237	BI421::[pBIO156] ₀	1,000	3,000
BI241	BI421::[Not/156] ₀	2,000	3,000

* Assayed using *E. coli* RY607 ($\Delta\text{bioABCD}$)

** Assayed using *E. coli* RY604 (ΔbioH)

Table 9

Biotin production by various <i>B. subtilis</i> strains containing integrated and amplified SP01-engineered <i>bio</i> operons.			
Strain Name	Relevant Features	Biotin (µg/l)*	Biotin & Vitamers (µg/l)**
PY79A	prototroph	10	10
BI421	<i>birA</i>	50	120
BI421	<i>birA</i>	80	150
BI267	PY79::[Not/158, 151] ₅₀	1,000	2,000
BI268	BI421::[Not/158,151] ₅₀	1,300	5,000
BI274	PY79::[Not/158,152] ₅₀	1,500	2,500
BI276	BI421::[Not/158,152] ₅₀	1,500	3,500

* Assayed using *E. coli* RY607 (Δ*bio*ABCDF)** Assayed using *E. coli* RY604 (Δ*bio*H)

Table 10

Biotin production by various <i>B. subtilis</i> strains.			
Strain name	Relevant features	Biotin (µg/l)*	Biotin & Vitamers (µg/l)**
PY79	prototroph	6	10
BI421	<i>birA</i>	45	200
BI239	amplified wild-type <i>bio</i> operon/ <i>birA</i>	580	2700
BI282	amplified SP01-15- <i>bio</i> operon/PY79	1100	3600

* Assayed with *L. plantarum str3*** Assayed with *S. cerevisiae*

VIII.B. Strain evaluation for large-scale biotin and biotin precursor production.

Engineered biotin-producing strains can be evaluated for large-scale biotin and biotin precursor production using fermentation technology. A range of fermenters and media conditions can be applied. As an example, all of the following fermentations were performed in computer controlled 14-liter Chemap fermenters utilizing a DO (dissolved oxygen) control, glucose-limited, fed batch fermentation strategy. The amount of biotin or biotin precursor produced was determined by inoculating serial dilutions of autoclaved cell-free broth with the appropriate strains of *Lactobacillus plantarum* or *Saccharomyces cerevisiae* as described above. The medium composition and other fermentation conditions are described in Table 11.

Biotin and biotin precursors produced by various strains are listed in Table 12. In all fermentations, 1 g/l pimelic acid was added to both the initial batch and feed solutions. BI282, BI278 and BI276 were the most optimized for biotin and biotin vitamer production.

Table 11. Biotin fermentation conditions and medium (VY) composition.

<u>Initial Batch</u>			
	<u>Grams</u>	<u>Volume</u>	<u>Time of Addition</u>
A. Veal Infusion Broth	150.00	4.5 liters	Sterilized for 60 mins. in fermenter 12.5 ml 50% NaOH pH 6.8 prior to sterilization, pH 6.6 post sterilization
Yeast Extract	30.00		
Sodium Glutamate	30.00		
KH ₂ PO ₄	45.00		
MgCl ₂ ·6H ₂ O	9.00		
MnSO ₄ ·H ₂ O	0.30		
FeCl ₃ ·6H ₂ O ₂	0.15		
(NH ₄) ₂ SO ₄	12.00		
MAZU DF37C	15.00		Approximately 800 ml volume gain
B. Glucose	150.00	0.3 liters	Added to fermenter immediately prior to inoculation
CaCl ₂ ·2H ₂ O	6.00		
<u>Feed Solution</u>			
C. KH ₂ PO ₄	54.70	0.2 liters	Added to D
MgSO ₄ ·4H ₂ O	6.00		
D. Glucose	3,000	3.3 liters	Combined with C and fed to fermenter
<u>Inoculum Medium</u>			
E. Inoculum Medium			Autoclaved separately Presterilization pH adjusted to pH 6.8
Composition = "A" + 0.35 gm CaCl ₂ ·2H ₂ O		300 ml	
F. 20% Maltose		50 ml	Added to E after cooling
G. 20% Glucose		25 ml	Added to E after cooling
<u>Acid</u>			
H. 3.5% H ₂ SO ₄		200 ml	Usual requirement for pH control
<u>Base</u>			
I. Anhydrous NH ₃			pH control

All solutions (A-G) sterilized separately and combined when cool.
Conditions Air: 1.5-2.0 vvm; RPM: 1000; pH 6.8; Temp. 37.0 °C; Pressure 0.6 bar.

Table 12

Biotin and vitamer production by first generation <i>B. subtilis</i> strains in bench scale fermenters.				
Fermentation Run ^a	Strain	Promoter/strain background	Biotin (mg/liter) ^b	Vitamins (mg/liter) ^c
B22 ^d	BI239	P _{bio} /BI421 (<i>birA</i>)	1	30
B19	BI268	SP01-26/BI421 (<i>birA</i>)	5	40
B23	BI276	SP01-26/BI421 (<i>birA</i>)	8	120
B20	BI278	SP01-15/PY79	10	60
B24	BI282	SP01-15/PY79	8	100

^aAll fermentations used VY salts medium (described in Table 11) with 1 g/liter pimelic acid in both batch and feed.

^bAssayed at 34 hours with *L. plantarum* str3.

^cAssayed at 34 hours with *S. cerevisiae*.

^dThe OD₆₀₀ of the culture was decreasing after 24 hours.

Table 13

Effect of complex nitrogen/nutrient source concentration on biotin and vitamer production by strain BI282 in bench scale fermenters.			
Fermentation Run	Medium ^a	Biotin (mg/liter) ^b	Vitamins (mg/liter) ^c
B30	1 X BY	10	150
B29	2 X BY	16	200
B31	3 X BY	8	130

^aFermentation conditions as described in Table 5, except with 1 g/liter pimelic acid in batch and feed, and with beef extract and proteose peptone substituted for veal infusion broth.

^bAssayed at 30 hours with *L. plantarum* str3.

^cAssayed at 30 hours with *S. cerevisiae*.

VIII.C. Analysis of fermentation broths by bioautography.

The spectrum of vitamins secreted by biotin-producing strains can be assayed by bioautography techniques. In the present case, fermentation broths were clarified by centrifugation and sterilized by autoclaving. One microliter aliquots of supernatant culture fluids were spotted on Baker-flex microcrystalline cellulose thin-layer chromatography (TLC) plates and the compounds were separated with a solvent of n-butanol and 1N HCl (6:1 v/v). After drying, the chromatograph was incubated for 1 hour, face down, on a biotin-free agar plate containing 2,3,5-triphenyltetrazolium chloride and kanamycin, and impregnated with *E. coli* strain RY604 (Δ bioH)/pOK12 (Kan^R). The Kan^R plasmid pOK12 [Viera and Messing, Gene 100, 189-194 (1991)] was added to RY604 merely to provide antibiotic resistance so that contamination could be reduced in the assay. The TLC plate was then removed and the agar plate incubated at 37 °C. After 20 hours, spots of growth corresponding to the location of biotin and vitamins on the TLC plates appeared. Fig. 15 shows biotin and vitamin standards with representative fermentation samples. The R_f values observed with this chromatography system are indicated in Table 14. The technique can also be employed using paper chromatography instead of cellulose TLC (Table 14). Comparison to bioautography utilizing RY634 (Δ bioA -

D-Kan^R, Bis⁺), which detects only biotin and biotin sulfoxide, indicated that substantial quantities of both desthiobiotin and biotin were present in the fermentation broth.

Addition of pimelate to the fermentation medium results in an increased level of a biotin vitamer which is probably KAPA (Fig. 15, lane D). This was shown by an increase in the KAPA:BSO spot compared to similar fermentations without pimelate (Fig. 15, lane B). Part of this material was demonstrated to be biotin sulfoxide, since it was also detected on bioautography utilizing RY634 (Δ *bioA*-*D*-Kan^R, Bis⁺) which detects only biotin and biotin sulfoxide. However, the intensity of the spot generated with RY634 at the KAPA:BSO location was significantly less than that detected with RY604:pOK12.

The accumulation of desthiobiotin and KAPA indicate limitations at the biosynthetic steps encoded by *bioB* and *bioA*. Such limitations may be overcome by elevated expression of these individual genes or by increases in the pools of substrates, cofactors or cooperating proteins for these steps. The expression of *bioB* or *bioA* can be separately elevated by either inserting subclones of the individual genes or PCR copies of the genes in an expression vector with a strong promoter (SP01, *veg*, etc.) and introducing the DNA into the cell on a plasmid such as pUB110, in a phage such as SP β , or integrated directly into a nonessential gene in the chromosome such as *bpr*.

Table 14

Observed and reported R_f values for biotin vitamers on cellulose chromatography		
Biotin vitamer	R_f	
	Observed, TLC	Literature ^a , Paper
DAPA	0.09	0.09
KAPA		0.35
Biotin sulfoxide	0.52	
Biotin	0.86	0.70
Desthiobiotin	0.94	0.82

^a Agric. Biol. Chem. 39, 779-784 (1975)

VIIID: Analysis of *bio*-specific mRNA synthesis in the engineered strains.

Northern blot experiments were performed on selected strains to examine the transcription pattern of the *bio* operon and the amount of *bio*-specific mRNA present in the various engineered strains. As expected a 7 kb RNA transcript covering the entire *bio* operon could be seen in all engineered strains. Lesser amounts of this transcript were also present in wild-type and *birA* mutant *B. subtilis* strains. In addition, all strains contained larger amounts (~8-fold) of a 5 kb transcript covering the first five genes in the *bio* operon, suggesting that a significant amount of transcript ended at a potential termination site after *bioB* (Fig. 5A). Significant amounts of a small transcript of 0.8 kb that covered most of the *bioW* gene were also seen. This transcript ended near a sequence with similarity to the consensus sequence of a site implicated in catabolite repression [Chambliss, G.H., "Bacillus subtilis and Other Gram-Positive Bacteria" edited by Sonenshein et al., Am. Soc. Microbiology, pp. 213-219 (1993)]. The relative ratio of the three transcripts to each other was the same in wild-type strains grown in the absence of biotin, *birA* mutant strains, or engineered strains driven by either the wild-type *bio* promoter or an SP01 promoter. Only the absolute amount of total *bio*-specific RNA varied dramatically in these strains. The engineered first generation production strains with a wild-type or an SP01-15 promoter produced about 30-fold or 60-fold, respectively, more *bio*-specific RNA than a derepressed wild-type cell. The *bio*-specific RNA levels in the *birA* mutant were only slightly (2-3 fold) higher than RNA levels in the derepressed wild-type cell, and were not affected by growth on biotin.

It appeared from these experiments that the SP01-promoters were directing the synthesis of at least 4-fold more RNA per operon than the wild-type *bio* promoter. However, with the reduced copy number of the SP01-*bio* operons, the total amount of *bio* specific RNA was at most only two-fold more than seen with a fully amplified, wild-type operon. The RNA levels correlated with biotin production levels, strains with the engineered, amplified SP01-*bio* operons produced about two-fold more biotin than *birA* mutant strains with the amplified wild-type operons, thus confirming that increasing the expression of one or more of the *bio* genes would lead to increases in biotin titer.

EXAMPLE IX. Genetic mapping of a *birA*-like gene of *B. subtilis*

In addition to cloned DNA that contains the *B. subtilis* *bio* operon, two recombinant plasmids, pBIO113 and pBIO114, were recovered which contained *B. subtilis* chromosomal DNA that complemented a temperature-sensitive mutation in the *birA* regulatory gene of *E. coli*. The *birA* gene product functions in *E. coli* both as an enzyme that catalyzes the addition of biotin to apoenzymes and as a repressor protein that negatively regulates expression of the *bio* biosynthetic genes.

The location of the *birA*-complementing gene on the *B. subtilis* chromosome was mapped by PBS1 generalized transduction. To do this, a *B. subtilis* bacterial strain was generated that contained a selectable antibiotic-resistance marker, *cat*, near the *birA* locus. Then, by determining the position of *cat* in the chromosome, the *birA*-complementing DNA was mapped. A derivative of pBIO113 was constructed that contained a *cat* cassette (obtained from pMI1101 [Youngman et al. *Plasmid* 12, 1-9 (1984)], and this integration vector was introduced into the *B. subtilis* chromosome. Since the 7.0 kb cloned insert of pBIO113 is homologous to its corresponding segment in the *B. subtilis* chromosome, integration of the *cat*-containing pBIO113 into the chromosome by Campbell recombination [Campbell, *Adv. Genet.* 11, 101-145 (1962)] introduced the *cat* gene near *birA*. Using standard PBS1-transduction mapping, the *birA*-complementing DNA was mapped to the 202⁺ region of the chromosome, very near the *trp* locus (> 90% linkage).

EXAMPLE X: Construction of a *B. subtilis* host strain deregulated for biotin production.**XA. Construction of Biotin analog-resistant strains**

Biotin analogs were used to select for strains that were deregulated for biotin production. Among the mutations sought were those in a potential homolog of the *E. coli* *birA* gene. However, it is expected that selection for resistance to biotin analogs can also yield strains with mutations in the operator site(s) of *birA*, or in genes encoding functions responsible for the transport of the analogs into the cell. Analog-resistant mutants can also contain a gene or genes that encode enzymes resistant to inhibitors including, but not limited to, feedback inhibitors. Biotin analogs (homobiotin, α -dehydrobiotin, and 5(2-thienyl)pentanoic acid) were obtained from Nippon Roche KK (Kanagawa, Japan). Mutagenized cells of *B. subtilis* were plated on TBAB (Difco Tryptose Blood Agar Base, cat. no. 0232-01-9) plates containing a crystal of each biotin analog.

B. subtilis PY79 (an SP β -cured prototroph derived from S.A. Zahler strain CU1769 (*metB5.glnA100*; Youngman et al. 1984 supra) was mutagenized with ethyl methane sulfonate (EMS) to give 90% killing. Surviving cells were grown overnight and 0.1 ml of culture was plated on TBAB plates. A crystal of two of the biotin analogs was placed on the plate and incubated overnight at 37°C. The α -dehydrobiotin and 5(2-thienyl) pentanoic acid crystals inhibited the growth of *B. subtilis* and gave zones of clearing around the crystals. Within the clear zones individual colonies appeared, providing likely candidates for biotin-analog resistant strains.

Several colonies were picked from the zones of clearing around the analogs α -dehydrobiotin and 5(2-thienyl) pentanoic acid and named DB-1 to DB-4 inclusive if selected from an α -dehydrobiotin zone, and TP-1 to TP-3 inclusive if selected from a 5(2-thienyl) pentanoic acid zone. The isolated colonies were streaked onto minimal-casamino acid plates with various amounts of each analog. All of these strains grew better (i.e., produced a larger colony) than wild-type cells on their respective analogs.

An additional 27 mutants were selected subsequently in similar plate screenings for their resistance to the analogs homobiotin (HB) and α -dehydrobiotin (α -DB). For this latter selection, *B. subtilis* PY79 cultures were subjected to mutagenesis with EMS in two independent experiments. The first mutagenesis, EMS1, resulted in 96% killing whereas the second, EMS2, resulted in 82% killing of the bacteria. Overnight cultures of PY79, EMS1, and EMS2 grown in rich medium were plated on TBAB (rich), BIOS (biotin free) or MIN (glucose-minimal) plates and a crystal of homobiotin or α -dehydrobiotin was placed on each of the plates. After 24 h, zones of killing were observed with a few resistant colonies growing within these zones. Individual colonies were picked from homobiotin plates or α -dehydrobiotin plates and restreaked on BIOS plates containing α -dehydrobiotin or homobiotin.

Potential repressor or operator deficient mutants were screened for their ability to secrete a measurable level of biotin. Each mutant strain was assayed for biotin and biotin vitamer production. Each strain was grown in VY medium (5 ml, 20 g/l Difco veal infusion broth and 5 g/l Difco yeast extract) for 18-24 hours at 37°C, and the supernatant was sterile-filtered. The filtered supernatants were then serially diluted in a biotin-free medium, and the serial dilutions were inoculated with *E. coli* strains RY604 (Δ *bioH*) and RY607

($\Delta bioABFCD$); the former grows on both biotin and biotin vitamers while the latter grows on biotin only. The biotin and biotin vitamers produced by different *B. subtilis* mutant strains were calculated from standard curves generated with biotin and desthiobiotin. Six mutants from the collection produced about 100 $\mu\text{g/L}$ of secreted biotin: homobiotin resistant mutants HB3, HB9, and HB15; and α -dehydrobiotin resistant mutants α -DB9, α -DB16, α -DB17. Other mutants produced either no biotin or 10 $\mu\text{g/L}$. Other mutants selected by the above method can be expected to provide 75 $\mu\text{g/L}$, 150 $\mu\text{g/L}$, or even 200 $\mu\text{g/L}$, 250 $\mu\text{g/L}$ or 300 $\mu\text{g/L}$.

XB: Mapping Biotin Analog Resistance Mutations

Example IX described mapping of the *B. subtilis* *birA* gene to a position just downstream of *trpC2*. The six biotin-secreting, analog-resistant mutants were examined for linkage to *trpC2* by phage transduction to determine if they are located in a *birA*-like repressor. Each candidate was crossed with *B. subtilis* 168 (*trpC2*), and Trp^+ transductants were patched to minimal plates with or without homobiotin. Results indicated that five of the six analog resistant mutations (HB3, HB9, HB15, α -DB16, and α -DB17) are closely linked to the *birA* locus (90%-95% co-transduction of Trp^+ and analog-resistance). BI421 is a homobiotin resistant (HB^r) Trp^+ transductant of strain 168 containing the *birA* mutation of α -DB16.

The sixth analog resistant mutation, that contained in α -DB9, was not linked to *trpC*, and therefore is not a single mutation at *birA*. By transducing from α -DB9 into a *bioW::cat7* strain in a similar transduction mapping experiment (see Figure 9), the mutation in α -DB9 was shown to be unlinked to the biotin operon. Therefore the mutant phenotype of α -DB9 is either due to a mutation at a third locus distinct from *birA* and from *bioWAFDBI*, or it is due to mutations at more than one locus, all of which are required to express the analog-resistant phenotype. The α -DB9 mutation can affect a biotin permease, a biotin export pump, or an enzyme related to biotin biosynthesis. Analog-resistant mutations that are at different loci (such as those of HB3 and α -DB9) can be combined in a single strain by standard strain construction techniques to give a strain with even greater capacity for biotin secretion. These analog-resistant mutants, or other mutants isolated and screened by the above procedures, may be used as host strains for biotin overproduction.

Two additional biotin analog-resistant mutations carried by α -DB12, isolated for resistance to α -dehydrobiotin as described above, and HB43, a spontaneous homobiotin-resistant mutant of PY79-*pbIO397cat*, were also mapped to the *birA* locus.

XC: DNA sequence of *B. subtilis* *birA* mutants.

Mutations resulting in amino acid changes can be found in the *birA* genes of homobiotin resistant strains such as HB3 and α -dehydrobiotin resistant strains such as α -DB16. To find such mutations, the DNA sequence of a wild-type *B. subtilis* *birA* gene can be compared with the DNA sequence of *B. subtilis* *birA* genes containing biotin analog-resistant mutations. The wild-type *B. subtilis* *birA* gene sequence can be obtained by sequencing the cloned *birA+* gene on pBIO113 or pBIO114. The mutant *birA* gene sequences can most easily be obtained from PCR copies of the gene. Several independent PCRs can be performed using genomic DNA from each *birA* mutant as template, and a pair of primers known to flank the *birA* coding region. DNA fragments can then be isolated from each independent PCR and cloned in *E. coli* pUC21 [Viora and Mossing Gene 100, 189-194 (1991)]. Isolates from each of two independent PCRs can be sequenced on both strands using a series of internal primers. Any artifactual mutations introduced by PCR should appear in only one of the two independent PCR clones, while the "true" mutation should appear in both independent isolates.

By comparison to the *E. coli* *birA* protein, for which the three dimensional structure is known [Wilson et al. Proc. Natl. Acad. Sci. USA 89, 9257-9261 (1992)], the mutations can be characterized. For example, the mutation may be located in one of the DNA-contacting helices. This information can be used to construct improved *birA* mutant strains of *B. subtilis* with reduced capacity to regulate expression of the *bio* operon. For example, two of the sequenced mutations could be combined in one gene using well known methods of site directed mutagenesis. Alternatively, small deletions that remove the DNA binding portion of *birA*, but not the biotin ligase activity can be constructed.

EXAMPLE XI. Second generation of engineered *B. subtilis* biotin production strains

While constructing and characterizing the first generation of engineered *B. subtilis* biotin production strains, applicants observed several limiting steps in the biotin regulatory system, modification of which can increase biotin production. For example, SP01 promoter-driven biotin operons were not amplified to as high a copy number as wild-type biotin operons, as shown by Southern blot data. First, identification of the gene

product that is deleterious to the cell when overproduced, and deletion of this gene from the amplified cassette, can circumvent this problem. Second, there are two points of partial pre-mature termination of mRNA synthesis in the biotin operon. The following Examples illustrate how this understanding of the biotin wild-type regulatory scheme can be used to optimize biotin production.

XIA. Constructions to improve the copy number of SP01 promoter-driven bio cassettes.

As shown above, biotin operons driven by SP01 promoters are less amplified than a biotin operon controlled by the wild-type operon. Applicants reasoned that the product of one or more of the *bio* genes is not tolerated at high levels. High level amplification can be achieved with a biotin operon lacking that specific *bio* gene. To determine which of these genes was not tolerated at high levels, Applicants designed the following experiment.

First, to assure some constitutive level of expression of all the *bio* genes, the biotin promoter was replaced in the chromosome with an SP01-15 promoter. To do this, an upstream homologous sequence was added to a 5' cassette containing the SP01-15 promoter (pBIO168). This construction was made by introduction of a 1.8 kb PCR fragment, generated from the sequence just upstream from the 5' biotin cassettes, into the *SalI* to *NruI* gap of pBIO168. The 1.8 kb PCR fragment was generated using primers designed to introduce a *NruI* site at the upstream, and a *SalI* site downstream, end. The resulting plasmid, pBIO180, has the SP01-15 promoter flanked by 1.8 kb of homologous sequence upstream of the *bio* operon and 0.7 kb downstream of the promoter. This plasmid was used to transform $\Delta::cat_1$ (see Example III), which has the promoter region of the biotin operon replaced by a *cat* cassette and is auxotrophic for biotin. A double recombination event allowed the replacement of the *cat* cassette with the SP01-15 promoter yielding the desired prototrophic strain, BI294 Cm^r.

A deletion in each of the *bio* genes can be generated by standard techniques. Below is one example of how a nonpolar deletion mutation was constructed in *bioW*.

A deletion of *bioW* was generated by altering the 5' cassette pBIO168. A PCR fragment was generated which has the SP01-15 promoter and first three codons of *bioW* followed by a *BamHI* site (Fig. 14). This PCR fragment was engineered so that after replacing the *XhoI* to *BamHI* fragment of pBIO168, the resulting 5' cassette, pBIO178, forms an in-frame *bioW* deletion upon ligation with the 3' *bio* cassettes (see Fig. 14). Transformation of this ligation mixture into *B. subtilis* BI294 (see above) and selection of Cm^r integrants (BI296) allowed amplification of the biotin operon without amplifying *bioW*. The chromosomal copy of *bioW* still transcribed from the SP01-15 promoter. A control with a complete SP01-15 driven biotin operon integrated into BI294 was also constructed and called BI295. Copy number of the operon was reduced in both cases, compared to amplified BI247 (Example VII Ciii). Comparison of the amplification of these two strains suggested that *bioW* is not the gene whose product is deleterious when overproduced.

This procedure can be repeated with each *bio* gene in turn to identify the gene that is deleterious when overproduced.

Analysis of biotin production by BI296, lacking the amplified *bioW* gene, compared to the isogenic strains BI295, containing an amplified *bioW* gene, indicated that the product of *bioW* is not the rate-limiting enzyme for biotin biosynthesis (Table 15A). BI296 produced about 10 times more biotin than the parent strains BI294 without the amplified *bio* cassette. Furthermore, BI296 produced similar amounts of biotin as BI295, the isogenic control strain with the complete SP01-*bio* operon. Repeating such experiments with internal, nonpolar deletions in each *bio* gene will identify the rate-limiting gene for biotin biosynthesis in *B. subtilis*.

Table 15A

Biotin production by various <i>B. subtilis</i> strains			
Strain name	Relevant features	Biotin ($\mu\text{g/l}$)*	Biotin & Vitamers ($\mu\text{g/l}$)**
PY79	prototroph	4	10
PY79	prototroph	6	10
DB16	<i>birA</i>	46	140
BI294	SP01- <i>bio</i> /PY79	165	360
BI294	SP01- <i>bio</i> /PY79	125	450
BI295A	amplified SP01- <i>bio</i> operon/BI294	1116	3150
BI295B	amplified SP01- <i>bio</i> operon/BI294	1405	5000
BI296A	ΔbioW amplified SP01- <i>bio</i> operon/BI294	1402	4000
BI296B	ΔbioW amplified SP01- <i>bio</i> operon/BI294	1574	3950

*Assayed with *L. plantarum* str3.

**Assayed with *S. cerevisiae*

XIB. Removal of possible transcription termination sites.

There are two internal sites of termination within the biotin operon. An mRNA fragment of about 0.8 kb is observed which corresponds to the distance from the promoter to a region in *bioW* which shares homology with the consensus sequence for a *B. subtilis* catabolite repression sequence (CRS). The major biotin transcript seen in Northern blots is 5.2 kb. This corresponds to the distance between the promoter and the *bioB-bioI* junction where a stem-loop structure is followed by a string of T residues. Constructions were made to eliminate the CRS and to increase the level of transcription past the *bioB-bioI* junction to the end of the operon.

XIBi: Removal of the catabolite repression sequence.

In *B. subtilis*, sporulation and the synthesis of certain enzymes are subjected to catabolite repression. [Chambliss, G. H., in "Bacillus subtilis and Other Gram-Positive Bacteria" Sonenshein et al., eds. Amer. Soc. Microbiology, Washington, D.C. pp. 213-219 (1993)].

Two potential catabolite repression sites (CRS) are located in or around the *bio* operon. One is located within the putative 5' leader region of ORF3. The second catabolite repression-like sequence was located within the 3' end of *bioW*. The location of this sequence coincides with the 3' end of a 0.8 kb -specific transcript detected in Northern blots, suggesting that catabolite repression might control, in part, expression of the *bio* operon. There is also a short AbrB regulatory sequence within this catabolite repression-like sequence [Stauch, M. A., in "Bacillus subtilis and Other Gram-Positive Bacteria", Sonenshein et al., eds. Amer. Soc. Microbiology, Washington, D.C. pp. 757-764 (1993)].

The portion of *bioW* encoding the *Bam*HI site and the CRS is illustrated in Fig. 17A. The CRS starts 11 bp downstream from the *Bam*HI site. Four codons in the sequence comprising the CRS can be converted to alternative codons by changes in the third position without altering the amino acid sequence. The third position changes alter three of the four most highly conserved residues (underlined) of the CRS (Fig. 17A).

As shown in Fig. 17A, the CRS site in *bioW* also has significant homology to an AbrB consensus binding site. A concern when altering the sequence of a CRS is that the binding site for AbrB is similar in sequence and care must be taken not to generate a strong AbrB binding site when destroying the CRS. However, the alterations introduced to destroy the CRS also reduce homology to the AbrB site. To introduce the changes indicated in Fig. 17A, a PCR primer was designed to include the *Bam*HI site, the CRS region with the desired mutations, and twenty residues for priming. An appropriate downstream primer allowed generation of a 660 bp fragment which could be digested with *Bam*HI and *Bst*1107I. Both *Bam*HI and *Bst*1107I restriction enzymes have unique sites in the plasmid pBIO289. The *Bam*HI and *Bst*1107I cut PCR product was cloned into *Bam*HI and *Bst*1107I cut pBIO289 to yield plasmid pBIO179. The *Bam*HI to *Eco*RI fragment from pBIO179 was then cloned into pBIO146A to generate a new 3' cassette plasmid, pBIO183. To change the sequence of the chromosomal copy of *bioW*, a two step protocol was utilized. First

a *cat* gene was introduced at the *Bam*HI site in *bioW* (see Example III) of BI294 (Example XIA). This generated an auxotroph BI294::cat7. Transformation of the auxotroph with linearized pBIO179 and selection for Bio⁺ yielded strain BI297 which has a single chromosomal copy of the biotin operon driven by the SP01-15 promoter with the sequence of *bioW* altered to destroy the catabolite repression sequence. The use of the new 3' cassette, pBIO183 with a 5' cassette i.e., pBIO168 to integrate and amplify in BI297 will assure amplification of a modified *bioW* and may relieve premature termination due to catabolite repression. This strain is BI306. With this procedure, second generation production strains are generated which might be less sensitive to catabolite repression.

10 XIBii: Removal or bypass of the termination site after *bioB*.

Two strategies were adopted to increase expression of biotin genes that lie downstream from internal sites of transcript termination. The first strategy involves deletion of the terminator. The second strategy is to insert an SP01-15 promoter in front of *biol*, in order to provide strong transcription of *biol* and ORF2.

To delete the terminator (Fig. 17B), which is in an intercistronic region, appropriate PCR primers were designed. One primer hybridized to *bioB*, upstream from a unique *Bsp*EI site. The second primer complemented the *Pml*I site, the ribosome binding site of *biol*, skipped 51 bp and then complemented the stop codon and 23 bp at the 3' end of *bioB* (Fig. 17B). Digestion with *Bsp*EI and *Pml*I generated a 209 bp fragment which was used to replace the *Bsp*EI to *Pml*I fragment of pBIO289 to give pBIO181. This plasmid was used to generate a new 3' cassette by cloning the *Bam*HI to *Eco*RI fragment into pBIO146A to yield pBIO185. Alteration of the chromosomal biotin operon in BI294 to delete the terminator between *bioB* and *biol* was accomplished in two steps. First, using a strategy similar to those described in Example III, a *cat* gene was introduced into the end of *bioB*. This yielded the Bio⁻, Cm^r strain BI300. When BI300 was transformed by linearized plasmid pBIO181, Bio⁺ isolates contained the desired terminator deletion and are represented by BI303. Integrated and amplified biotin operons containing the terminator deletion were constructed by transforming BI303 with ligated *Bam*HI to *Not*I fragments from pBIO168 and pBIO185 and are represented by BI307.

To assure maximum expression of *biol* and ORF2, an SP01-15 promoter was introduced in front of *biol*. The SP01-15 promoter from pBIO168 was amplified by the PCR, introducing the ribosome binding site and start codon/*Pml*I site of *biol* on the downstream side, and a *Stu*I site on the upstream side. Primers used were: 1) 5'-GGC CAT TCT ACA CGT GAT TTT CTC CTT TCT GTC TAG AAC AGG CGG GGT TGC; and 2) 5'-GGC CAG GCC TGG CTA TTG ACG ACA GCT ATG GTT. Since digestion of DNA by *Stu*I and *Pml*I creates blunt ends, digestion of pBIO289 with *Pml*I allowed introduction of the *Stu*I/*Pml*I digested PCR fragment. In one orientation the *Pml*I site is regenerated at the *biol* start codon and the SP01-15 promoter directs transcription of *biol* and ORF2. The plasmid with this orientation was called pBIO182. A new 3' cassette (pBIO184) was constructed by cloning the *Bam*HI to *Eco*RI fragment of pBIO182 into pBIO146A. This construction is expected to generate even more transcription of *biol* and ORF2 than would be generated by elimination of termination between *bioB* and *biol*.

The SP01-15 driven *biol* construction was introduced into the chromosomal copy of BI294 by transduction of BI300 (see above) with linearized pBIO182 and selection for Bio⁺ yielding BI304. Integration and amplification gave BI308.

XIBiii: Biotin production by single copy terminator modified strains.

Biotin and vitamers were assayed from test tube cultures as described in Example VIIIA utilizing *Lactobacillus* and *Saccaromyces*. BI294 was used as a control for BI303 which deleted the terminator between *bioB* and *biol* and for BI304 which introduced a SP01-15 promoter before *biol*. As demonstrated in Table 15B, deletion of the terminator or introduction of the SP01-15 promoter before *biol* have little effect on biotin titers but dramatically increase the production of biotin vitamers.

Table 15B

Biotin and Vitamer Assays of Terminator Modified Strains.			
Strain	biotin locus	Biotin $\mu\text{g/l}$	Vitamer $\mu\text{g/l}$
BI294C	SP01-15 <i>bio</i>	126	481
BI294D		315	528
BI303A	SP01-15 <i>bio</i> Δ T	313	838
BI303B		200	790
BI304A	SP01-15 <i>bio</i>	182	2800
BI304B	SP01-15 <i>bioI</i>	179	3138

EXAMPLE XIX: Altering *bio* gene ribosome binding sites.

Translation of genes in the *bio* operon can be improved by altering the ribosome-binding sites to conform more closely to a canonical *B. subtilis* ribosome binding site with the sequence 5'AGAAAGGAGG-TGA3'. Such changes can be introduced by synthesis of a DNA primer encoding an appropriate restriction site, the modified ribosome-binding site, and sufficient downstream DNA to insure priming of a PCR reaction. By selection of an appropriate second primer, one skilled in the art can synthesize a PCR product containing the modified ribosome-binding site. This PCR fragment containing the altered ribosome-binding site can then be introduced into an engineered *bio* operon by the same methodology used to introduce the modified CRS sequence described in Example XIBi.

EXAMPLE XII: Azelaic acid-resistant (Azl^r) mutants of *B. subtilis*.

Azelaic acid, a straight chain C₉ dicarboxylic acid, is a homolog of pimelic acid and is thought to be an intermediate in the conversion of oleic acid to pimelic acid [see Ohsugi and Inoue *Agric. Biol. Chem.* 45, 2355-2356 (1981)]. Pimelic acid at 1 g/l can stimulate biotin vitamer production in *B. subtilis* and pimelic acid at 30 mg/l can restore wild-type growth to a PY79 *bioI::cat₃* bradytroph (see Example III). Azelaic acid at 30 mg/l does not substitute for pimelic acid in supporting growth of PY79 *bioI::cat₃*. In fact, azelaic acid at 30 mg/l severely inhibited the growth of PY79 *bioI::cat₃*. Azelaic acid at higher concentrations inhibits the growth of wild-type *B. subtilis*, this inhibition being reversed by addition of 1 $\mu\text{g/l}$ biotin. From these results, Applicants reasoned that azelaic acid is a specific inhibitor of biotin biosynthesis in *B. subtilis*.

A wild-type *E. coli* strain, MM294, is relatively resistant to azelaic acid. The *E. coli* strain RY604- (Δ *bioH*), containing pBIO403 which includes only the *bioW* gene from *B. subtilis*, is auxotrophic for biotin, although 30 mg/l pimelic acid can satisfy the biotin requirement. However, RY604/pBIO403 grown in the presence of excess pimelic acid (80 mg/l) is sensitive to inhibition by azelaic acid. Therefore, Applicants concluded that azelaic acid acts at the level of pimelyl CoA synthetase (*bioW*), either as a competitive inhibitor of pimelic acid, or by incorporation into a biotin homolog or other toxic intermediate.

XIIA: Isolation of azelaic acid resistant mutants of *B. subtilis*.

On minimal agar, azelaic acid at 2 g/l (about 10^{-2} M) severely inhibited growth of PY79, although it did not kill or completely prevent growth. Seven spontaneous mutants that outgrew the background of PY79 on 2 g/l Azelaic acid were isolated. The resistance to azelaic acid appeared to be a stable trait in all but one case (see below). The seven mutants were provisionally named PA1 - PA7 (PY79 Azelaic acid resistant). PA1 through PA7 were grown in test tubes in VY medium, and biotin production was assayed using *E. coli* indicator strains (Table 15). The mutants fell into two classes, those that yielded more biotin than PY79 (PA5, PA6) and those that were similar to PY79 (PA1, 2, 3, and 7). PA4 appeared to be either unstable or not a true mutant and was dropped from further study. Applicants also noticed that the mutants fell into two classes with respect to colony size on minimal agar with 2 g/l azelaic acid, and that these two classes corresponded to the two classes of biotin producers (Table 11). The mutants with the most biotin in the supernatant (PA5 and PA6) gave small colonies, while the biotin non-secreters, PA1, 2, 3, and 7, gave large colonies. PA1 and PA3 secreted a compound that cross-fed PY79 (i.e., reversed the azelaic acid inhibition

of PY79) on minimal plates containing 2 g/l azelaic acid

Representatives of the two classes of azelaic acid resistant mutants, PA3 and PA6, were chosen for further characterization. In liquid minimal cultures containing serially diluted azelaic acid, PA3 and PA6 showed clear resistance to azelaic acid compared to the parent, PY79 (Fig. 18). However, the dose response curves of PA3 and PA6 were distinct from each other. PA3 showed greater resistance than PA6, and at lower concentrations of azelaic acid PA6 did not grow to the same cell density as PA3 or PY79

XIIB: Mapping of azelaic acid resistant mutants.

To map the azelaic acid resistant mutations in PA3 and PA6, Applicants determined whether either mutation maps at *birA* or at the biotin operon. In the first case, PBS1 transducing lysates from PA3 and PA6 were applied to strain RL1 (*trpC2*), and Trp⁺ transductants were selected. The *trpC2* and *birA* loci are about 90% linked by transduction. The Trp⁺ transductants were then screened for azelaic acid resistance by patching to minimal agar containing 2 g/l azelaic acid. No Trp⁺ transductants were azelaic acid resistant, demonstrating that neither mutation is linked to *trpC2*, and therefore neither is a *birA* mutation. The PA3 mutation showed strong linkage to *bio* in two transductions (one into PY79 *P_{bio}::cat17* and one into PY79 *bioW::cat7*), while the PA6 mutation did not.

XIIC: Effect of azelaic acid resistance mutations on biotin production.

Another approach to altering biotin regulation is to combine either of the azelaic acid resistant mutations with a *birA* mutation.

The *birA* mutation from either HB3 or α -DB16 was introduced into either PA3 or PA6 by a two step transduction process. First, PA3 and PA6 were made Trp⁻ by transduction with *trpE::Tn917lac* [Perkins and Youngman, *Proc. Natl. Acad. Sci. USA* 83, 140-144 (1986)] selecting for erythromycin resistance. The *birA* mutations were transduced from HB3 or α -DB16, selecting for Trp⁺. Parents and Trp⁺ transductants were screened for homobiotin resistance and azelaic acid resistance. PA6 was homobiotin sensitive, so double mutants could be identified among Trp⁺ transductants by screening for homobiotin resistant colonies. Only about 60% of Trp⁺ transductants were also homobiotin resistant (this may be due to Tn917 distortion of the map distance between *birA* and *trpE* normally 90% linkage by transduction). PA3 was resistant to homobiotin, so direct identification of double mutants was not possible. However, 60% of the transductants were double mutants as judged by increased biotin secretion, see below.

The parent strains, putative PA3 *birA* double mutants, and actual PA6 *birA* mutants, were tested for biotin and vitamin production in VY test tube cultures. As shown in Table 18, the double mutants derived from PA3 produced about four to six fold more vitamin and twice as much biotin as the *birA* parent. Double mutants derived from PA6 produced similar or only slightly more biotin and vitamin than the *birA* parent. Clearly, the PA3 mutation aids biotin production in a deregulated strain.

XIID: Additional azelaic acid resistant mutants.

In addition to the azelaic acid resistant mutants of the types represented by PA3 and PA6, several other azelaic acid resistant mutants that represent at least two new classes have been isolated as spontaneous mutants from the PY79 strain background.

Eleven additional mutants were partially mapped by transduction as described above to determine if the azelaic acid resistant mutation was linked to *birA* or to the *bio* operon. None were linked to *trpC2*, so by inference, none were at the *birA* locus. On the other hand, eight of the eleven tested were linked to the *bio* operon. Of those eight, two were tightly linked to the *bio* promoter, as was PA3, while six were substantially less than 100% linked to the *bio* promoter, suggesting that the mutations were in the *bio* operon well downstream from the promoter. Thus this latter group of six mutants represents another class of azelaic acid resistant mutants distinct from PA3 and PA6. This group includes BI514, BI521, BI532, BI535, BI537 and BI545. This group of mutants is likely to include mutants that have increased capacity to produce or utilize pimelic acid, for example *bioI* or *bioW* mutants.

Three of the new mutants did not map at *birA* nor at the *bio* operon. This group includes BI523, BI544, and BI549, and is likely to contain mutants that produce increased levels of pimelic acid precursors or that are more efficient at converting various biotin precursors into biotin. None of this group were equivalent to PA6, since unlike PA6, they all grew to the same density as PY79 (wild type) in a minimal medium lacking azelaic acid.

The new mutants are summarized in Table 19. Although none lead to significantly increased biotin production by themselves, these mutations are likely to increase biotin production when combined with other biotin deregulating mutations as was the case for PA3.

Applicants have deposited on May 4, 1994 under the terms and conditions of the Budapest Treaty strains PA3, HB43, HB3, BI544, BI535, BI421, BI304, BI282 and BI274 with the American Type Culture Collection (ATCC) in Rockville, Maryland, USA and they have received accession numbers ATCC 55567, 55568, 55569, 55570, 55571, 55572, 55573, 55574 and 55575 respectively.

Table 17

Biotin and vitamer production by azelaic acid resistant mutants in test tube cultures.			
Strain	Colony size on 2 g/l azelaic acid	Biotin ($\mu\text{g/l}$)	Vitamins ($\mu\text{g/l}$)
PY79	tiny	10	11
PA1	large	10	13
PA2	large	10	12
PA3	large	10	12
PA4	tiny	10	11
PA7	large	10	12
PA5	small	30	60
PA6	small	30	60
VY Medium (no cells)	-----	30	100

Table 18

Biotin and vitamer production by birA ^{azl} double mutants.					
Parent strain	Donor for <i>birA</i> gene	Isolate number	Homobiotin resistant/sensitive	Biotin: ^a (μg/l)	Vitamins ^b (μg/l)
VY medium	----		----	22	25
PY79	----		S	6	10
PA3	----		R	5	9
PA3	HB3	1	R	120	690
PA3	HB3	2	R	100	440
PA3	α-DB16	1	R	100	690
PA6	----		S	11	16
PA6	HB3	2	R	46	200
PA6	HB3	6	R	50	120
PA6	α-DB16	8	R	53	220
PA6	α-DB16	14	R	56	200
HB3	----		R	44	110
α-DB16	----		R	46	140

a. Assayed using *L. plantarum* str3b. Assayed using *S. cerevisiae*

Table 19

Additional Azelaic Acid Resistant Mutants	
Strain Name	Linkage to P_{bio}^a
BI530	100
BI533	100
BI514	70
BI521	90
BI532	80
BI535	40
BI537	40
BI545	90
BI523	0
BI544	0
BI549	0
PA3	100
PA6	0
PY79 (wild type)	-

^aApproximate linkage in percent BIO⁺, azelaic acid resistant upon PBS1 transduction into PY79 $P_{bio}::cat17$

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: BIOTIN BIOSYNTHESIS IN BACILLUS SUBTILIS

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: System 7.1 (Mac)
 (D) SOFTWARE: Microsoft Word 5.0

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/084,709
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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8478 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGCATCGGAG ATCCAAAGCC TGATCGCGCC GCGCCCGCAC CTTAGTCTTG TTGGTGTACA 60
 CGATCGGTTA ACGCCGGCTG AGGGCGTGGA CAAAATCGAA AAAGAATTGA CAGCTGTCTA 120
 TGCTGGACAG GGAGCTGCTG ATTGCTACCG AGTGGTCCGT TCTGCTTCGG GACATTTCTA 180
 AACAGCAGTT ATAAGGCATG AAGCTGTCCG GTTTTTCGAA AAGTGGCTGT GACTGTAAAA 240
 AGAAATCGAA AAAGACCGTT TTGTGTGAAA ACGGTCCTTT TGTTTCCTTT TAACCAACTG 300
 CCATAAATCG ATCCTTTCTT CTATTGACAG AACAGGAGA GAATAATATA TTCTAATTGT 360
 TAACCTTTGA ATATAATTGG TTAACAATTT AGGTGAGAAG CGCTACACGT TCTTCAGTTA 420
 TCAGTGAAAG GCGGAGAAAT GATGCAAGAA GAAACTTTTT ATAGTGTGAG AATGAGGGCT 480
 TCAATGAATG GATCTCATGA AGACGGCGGA AAGCATATAT CCGGCGGAGA ACGGCTTATT 540
 CCTTTCATG AGATGAAGCA TACAGTCAAT GCTTTATTAG AAAAAGGGTT ATCCCATTCA 600

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AGAGGAAAAC CTGATTTTAT GCAAATTCAA TTTCAGAGG TACATGAATC GATAAAAACC 660
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 5 GCGCGATTGT TATTGGAAAA AGAAGGCGTT TCACGAGACG TGATTGAAAA AGCATATGAA 780
 CAAATCCCTG AATGGTCAGA TGTCAAGGGT GCGGTCTTGT TTGATATTCA TACAGGCAAG 840
 CGAATC ATC AAACAAAAGA AAAAGGGGTG CCGGTCTCCA GAATGGATTG GCGGACGCT 900
 10 AATTTTGAAA AATGGGCGCT TCACAGTCAC GTGCCAGCTC ATTCAAGAAT AAAAGAGGCC 960
 CTTGCGCTCG CTTCAAAGGT AAGCCGGCAC CCGGCAGTCG TTGCAGAATT ATGCTGGTCG 1020
 GACGATCCGG ATTACATAAC AGGCTATGTT GCGGTAAGA AAATGGGCTA TCAGCGTATT 1080
 15 ACAGCAATGA AAGAATACGG GACTGAAGAG GGCTSCCGAG TCTTTTTTAT TGATGGATCC 1140
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	AGCGATCCAC GAGGTTACGA GCCTTGAAGA TTGATTCCTG GTTAAACGAG CGGTTAGACA	2550
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5	AGAGGAATAT TGATGGCGAA AATCAAAACGG TCTGGTCCTC AAACAATTAT TTAGGGCTCG	2700
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15	TTGATATGAA TGATCTTGAA AACAAAGCTGA ATGAAACACA GCGTTATCAG CGCCGTTTTA	3060
	TCGTAACAGA CGGAGTATTC AGCATGGATG GCACAATCGC CCCTCTTGAT CAGATCATCT	3120
	CACCTGCGAA ACGCTATCAT GCCTTCGTGG TCGTTGATGA TGCCACGCA ACAGGAGTTT	3180
20	TGGGCGATTG GGGACAAGGA ACGAGTGAAT ACTTTGGTGT TTGTCCCGAC ATTGTTATCG	3240
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	TCGACTTTTT GCTGAACCAT GCCAGACAT TTATCTTTCA AACCGCTATT CCGCCAGCCA	3360
25	GCTGTGCGGC TGCTCAGAG GCTTTAACA TCATTGAAGC CAGCAGGGA AAACGACAGC	3420
	TTTTATTTTC TTATATCAGC ATGATCAGAA CCAGTCTGAA GAATATGGGT TATGTGGTGA	3480
	AAGGAGATCA CACACCGATT ATTCTGTAG TCATTGGCGA TGCCCATAAA ACGGTCTTAT	3540
30	TTGCTGAAAA ACTGCAGGGC AAGGGAATTT ATGCTCCTGC CATTCCGGCG CCAACCGTTG	3600
	CGCCGGGTGA AAGCCGGATT CGAATTACAA TCACGTCTGA CCACAGTATG GGTGATATTG	3660
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35	TTTTGTGACG GGAAGTATA CAGAAGTAGG GAAAACGGTT ATATCCAGCG GTCTTGCTGC	3780
	CTTATTGAAA GACAATAATA GACATGTCGG GGTGTATAAA CCATTTTAA GCGGGATATC	3840
	GCGCCATCAT CCAGATAGTG ATACAAAGTT GCTGAAAGAT ATGTCCGAGA CCAGTCTTTC	3900
40	TCATGAAGAC ATTACGCCCTT TTGCCCTCAA GGCGCCGCTT GCACCATACG TTGCAGGGAA	3960
	ACTTGAGGGA AAGACTGTCA CCATGGAAGA GGTTTTAAGC CATTGGGGGC GGATTAGAGA	4020
	AAAACATGAA TGCTTCATCG TAGAAGGTGC AGGCGGTATT TCTGTGCCAT TGGGAGAGGA	4080
45	CTATTTGGTC AGTCATGTCA TAAAAGCGTT GCAGCTTCCC ATGATTATTG TGGCGCGTCC	4140
	TCGCCTTGGA ACCATTAATC ATACCTTTTT AACTGTCAAA TATGCAGAAA GCATGGGGCT	4200
	TCCAATCGCC GGAATTATCA TCAATGGAAT CAGTACTCT CCTGATGAAG ATGAAAAAAC	4260
	CAATCCTGAG ATGATTGAGC GCTTATGCGG TGTCCCGATT TTAGGGGTTA CGCCAAAGCT	4320
50	TGCCAACGTG ACGAAAGAAA CGCTTCTACA TATGCTAAAA GACCATATCA ATCTATCATT	4380

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	ACTGATGAAT CAAGTGGGGG TATGAGAATG AATCAATGGA TGGAACTCGC AGACCGGGTG	4440
	CTGGCTGGAG CAGAAGTGAC TGACGAAGAG GCGCTTTCAA TATTACATTG TCCTGATGAA	4500
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	GTAAASCTCA ATATGATTAT GAATGCGAAA TCCGGGCTCT GCCCGGAAAA CTGCGGCTAT	4620
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10	ACGCTGCTTG AAGGCGCGAA GCGGGCGCAC GATCTGAATA TCGGCACATA TTGTATCGTG	4740
	GCAAGCGGCA GAGGTCCGTC TAACAGAGAA GTGGATCAGG TCGTAGATGC GGTTCAGGAA	4800
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15	GCGAAGCGGC TCAAAGATGC AGGAGTAGAC CGCTATAATC ATAATTTGAA TACGTCACAG	4920
	AGAAACCATT CAAACATCAC AACCTCACAT ACATACGATG ACAGAGTCAA TACGGTTGAA	4980
	ATCGCAAAAG AATCGGGGCT GTCTCCGTGT TCAGGCGCCA TTATCGGGAT GAAGGAGACG	5040
20	AAACAGGATG TCATTGACAT CGCCAAAAGC TTGAAGGCTC TTGACGCGGA TTCCATTCTT	5100
	GTGAATTTTT TGCATGCAAT TGATGGCAGC CCGTTAGAAG GCGTCAACGA ATTAACCCG	5160
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	GTTTCTTAAA ATACCCGGGC TGGTATGTCA CAGGATATGA AGAAACGGCT GCTATTTTGA	5640
35	AAGATGCGAG ATTCAAAGTC CGCACCCCGC TGCCTGAGAG CTCAACCAAA TATCAGGACC	5700
	TTTCACATGT GCAAAATCAA ATGATGCTGT TTCAGAACCA GCCTGATCAT AGACGATTGC	5760
	GGACGCTTGC CAGCGGAGCG TTTACGCCGA GAACGACAGA GAGTTATCAG CCGTATATCA	5820
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	CGGACTTTGC TTTTCCTTTA GCAAGTTTTG TCATAGCTAA CATTATAGGT GTACCGGAGG	5940
	AAGATAGGGA GCAATTAAG GAGTGGGCTG CGAGTCTCAT TCAAACGATT GATTTTACCC	6000
45	GCTCAAGAAA GGCATTAACA GAGSGCAATA TTATGGCTGT GCAGGCTATG GCATATTTCA	6060
	AAGAGCTGAT TCAAAAGAGA AAACGCCACC CTCACAGGA TATGATCAGC ATGCTCTTGA	6120
	AGGGGAGAGA AAAGGATAAG CTGACGGAAG AGGAGGCGGC ATCTACGTGC ATATTGCTGG	6180
50	CGATCGCCGG ACATGAGACA ACGGTCAATC TCATCAGCAA TTCAGTCCTT TGTCTGCTGC	6240
	AGCATCCAGA ACAGCTTTTG AACTGAGAG AAAATCCAGA TCTTATTGGT ACCGCAGTCG	6300

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	AGGAATGTTT ACGCTATGAA AGCCCCACGC AAATGACAGC CAGAGTTGCG TCAGAGGATA	6360
	TTGACATCTG CGGGGTGACG ATCCGTCAAG GAGAACAAGT CTATCTTTTG TTAGGAGCGG	6420
5	CTAATCGAGA CCCTAGCATA TTCACGAACC CCGATGTCTT CGATATTACG AGAAGTCCTA	6480
	ATCCGCATCT TTCATTCGGG CATGSCCATC ATGTTTGCTT AGGGTCCTCG CTGSCACGAT	6540
	TAGAAGCGCA AATTGCGATT AACACTCTTC TGCAGCGAAT GCCCAGCCTT AATCTTGCGG	6600
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	CAGATCGGCC ATTTGTTTTA TTTGGAAAAT AAGCTATCAG TAATCAACCT TCGAATCGGA	7260
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5 TGATCATTCT GAAAAACTTC TTTCAAAATA TCCCGTCAAG CCTGGAAGAG TCCGCGAAAA 8220
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 10 AGCAAACCAT NNNNNTGG 8478

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTGACANNNN NNNNNNNNNN NNNTATATT 29

25 (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

35 TTGACANNNN NNNNNNNNNN NNNTATAAT 29

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTGTAANNNN NNNNNNNNNN NNNNTAATAT 30

50 (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

55

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTGATANNNN NNNNNNNNNN NNNAAAAGT 29

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTGAAANNNN NNNNNNNNNN NNNTCTTAT 29

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 300 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAGCTGTCCG GTTTTTGCAA AAGTGGCTGT GACTGTAAAA AGAAATCGAA AAAGACCGTT 60
TTGTGTGAAA ACGGTCTTTT TGTTTCCTTT TAACCAACTG CCATAAATCG ATCCTTTCTT 120
CTATTGACAG AAACAGGAGA GAATAATATA TTCTAATTGT TAACCTTTGA ATATAATTGG 180
TTAACAATTT AGGTGAGAAG CGCTACACGT TCTTCAGTTA TCAGTGAAAG GGCGAGAAAT 240
GATGCAAGAA GAAACTTTTT ATAGTGTGAG AATGAGGGCT TCAATGAATG GATCTCATGA 300

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AATGTGTTAA CTTAAAAACT ATAGTTGGTT AACTAA 36

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTAATTGTGA ACCTTTGAAT ATAATTGGTT AACAATTTAG 40

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGCCAAGCTT GTCGACCGAA ACAGCAGTTA TAAGGCAT 38

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGCCCGTCTA GAGCTTCTCA CCTA 24

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGCCGAGAAG CTCTAGACGT TCTTCAGTTA TCACT 35

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGCCAGGGTT TTCCAGTCA CGAC 24

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TAGAAGAAAG GCTCGAGTTA TGGCAGTT 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AACTGCCATA ACTCGAGCCT TTCTTCTA 28

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Ala Val Arg Phe Leu Gln Lys Trp Leu
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Met Met Gln Glu Glu Thr Phe Tyr Ser Val Arg Met Arg Ala Ser Met
 1 5 10 15
 Asn Gly Ser His Glu
 20

Claims

1. A DNA comprising a DNA sequence that is selected from the group consisting of:
 (a) a DNA sequence of a gene that encodes a biotin biosynthetic enzyme of *Bacillus subtilis*, or of a species closely related to *Bacillus subtilis*;
 (b) a DNA sequence of a biologically active fragment of (a); and
 (c) a DNA sequence that is substantially homologous to (a) or (b).
2. The DNA of claim 1, wherein said gene is selected from the group consisting of *bioA*, *bioB*, *bioD*, *bioF*, *bioW*, *bioI*, and ORF2.
3. The DNA of claim 1, wherein said gene is *bioA* of *B. subtilis*, or a closely related species thereof.
4. The DNA of claim 1 wherein said gene is *bioB* of *B. subtilis*, or a closely related species thereof.
5. The DNA of claim 1, wherein said gene is the *bioI* gene of *B. subtilis*, or a closely related species thereof.
6. The DNA of any of claims 1-5, wherein said DNA sequence is operably linked to a transcriptional promoter.
7. The DNA of any of claims 1-6, wherein said DNA comprises at least two of said DNA sequences.
8. The DNA of claim 7, wherein a first one of said DNA sequences is operably linked to a first transcriptional promoter, and a second one of said DNA sequences is operably linked to a second transcriptional promoter.
9. The DNA of claim 8, wherein at least one of said promoters is a constitutive promoter.
10. The DNA of claim 9, wherein said constitutive promoter is derived from the SP01 bacteriophage.
11. The DNA of claim 8 wherein said first one of said DNA sequences is the one of a gene selected from the group consisting of *bioA*, *bioB*, *bioD*, *bioF*, and *bioW* of *B. subtilis*, or a closely related species thereof; and said second DNA sequence is the one of a *bioI* of *B. subtilis*, or a closely related species thereof.
12. The DNA of claim 8 or of claim 11 wherein at least the following DNA sequence(s) is operably linked to said second promoter: said *bioA*, said *bioB*, or both said *bioA* and said *bioB*.
13. The DNA of claim 6 wherein said DNA comprises the DNA sequence of the biotin operon of *Bacillus subtilis*, or a closely related species thereof, operably linked to said transcriptional promoter.

14. The DNA of any of claims 1-13 comprising a regulatory site of a biotin operon of *B. subtilis* or a closely related species, said regulatory site being mutated with respect to wild-type DNA and being selected from the group consisting of an operator, a promoter, a site of transcription termination, a site of mRNA processing, a ribosome binding site, and a site of catabolite repression, said mutation being either an insertion, a substitution, or a deletion.
15. A vector comprising a DNA as claimed in any one of claims 1-14.
16. A cell comprising the vector of claim 15 or a DNA as claimed in any one of claims 1-14.
17. The cell of claim 16, wherein said DNA is amplified to multiple copies in said cell.
18. The cell of claim 16, wherein said DNA is stably integrated into the chromosome of said cell.
19. The cell of claim 18, wherein said DNA is amplified to multiple copies in said chromosome of said cell.
20. The cell of claim 18 or claim 19, wherein said DNA is integrated at the *bio* locus of said chromosome.
21. The cell of any of claims 18-20, wherein said DNA is integrated at more than one site in said chromosome.
22. The cell of any one of claims 16-21, wherein said cell is characterized by a mutation that deregulates production of biotin or a biotin precursor, in addition to the presence of said DNA.
23. The cell of any one of claims 16-22 wherein said cell produces an increase in biotin in comparison to wild-type cells lacking said DNA.
24. The cell of claim 22, wherein said cell contains a mutation that confers resistance to azelaic acid.
25. The cell of claim 22, wherein said mutant cell is mutated in *birA*.
26. The cell of any one of claims 16-25, wherein said cell is *Bacillus subtilis*, or a closely related species thereof.
27. The cell of any one of claims 16-25, wherein said cell is *Escherichia coli*.
28. A recombinant protein encoded by a DNA as defined in any one of claims 1-5.
29. A process for the production of biotin or a precursor thereof, said process comprising the steps of:
 - (a) providing the cell of any one of claims 16-27;
 - (b) culturing said cell for a time and under conditions which allow synthesis of biotin or said precursor, and
 - (c) isolating said biotin or said precursor.
30. The process of claim 29, wherein said biotin or said precursor is secreted from said host cell and isolated from the extracellular media of said host cell.

FIG. 1

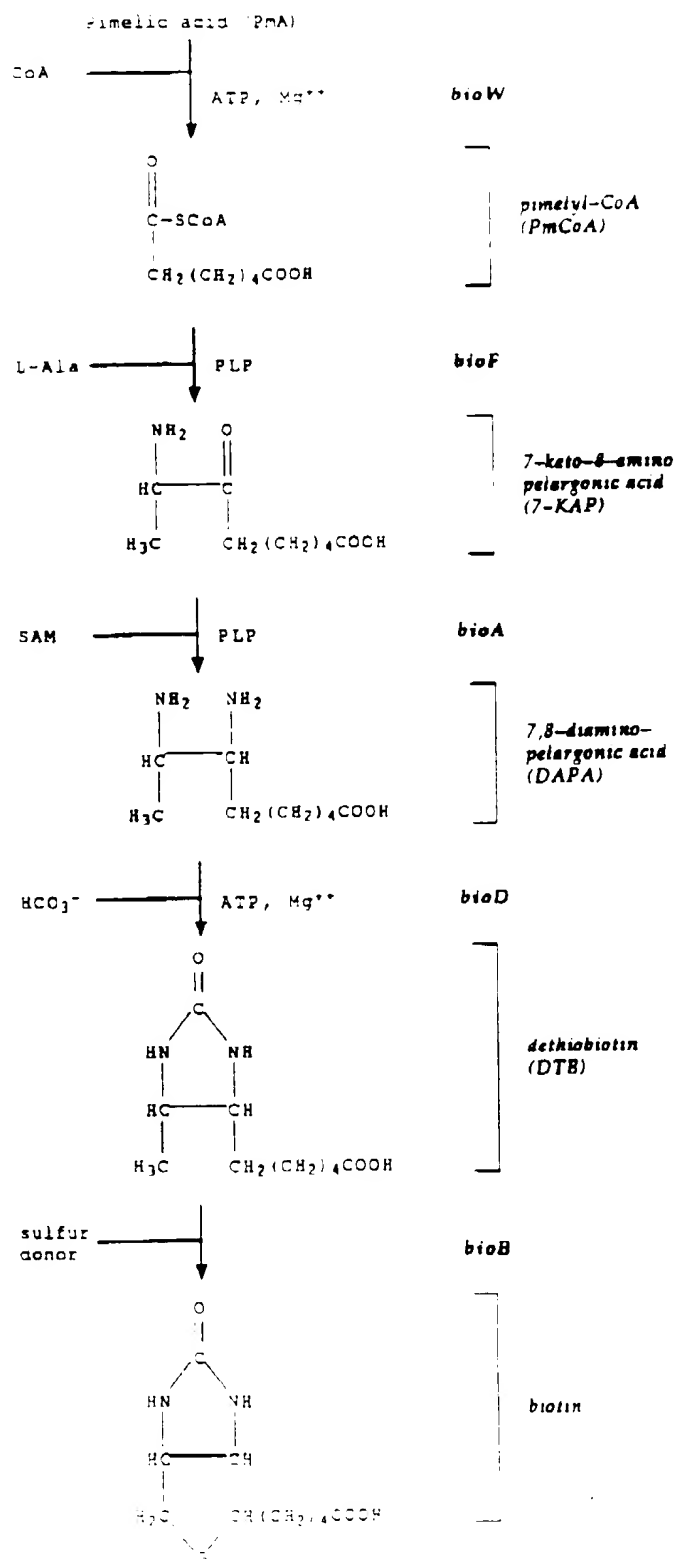


FIG. 2

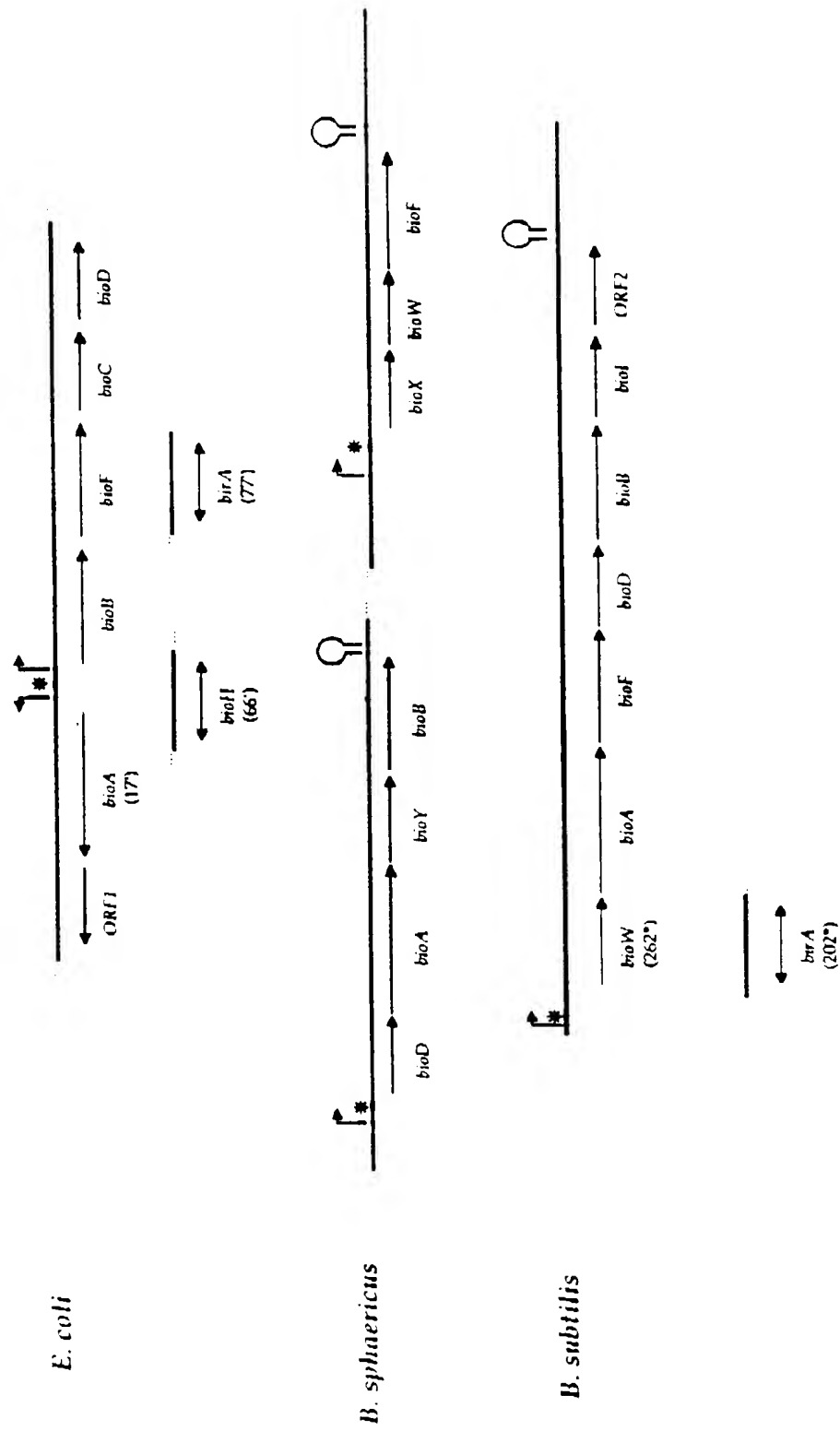


FIG. 3

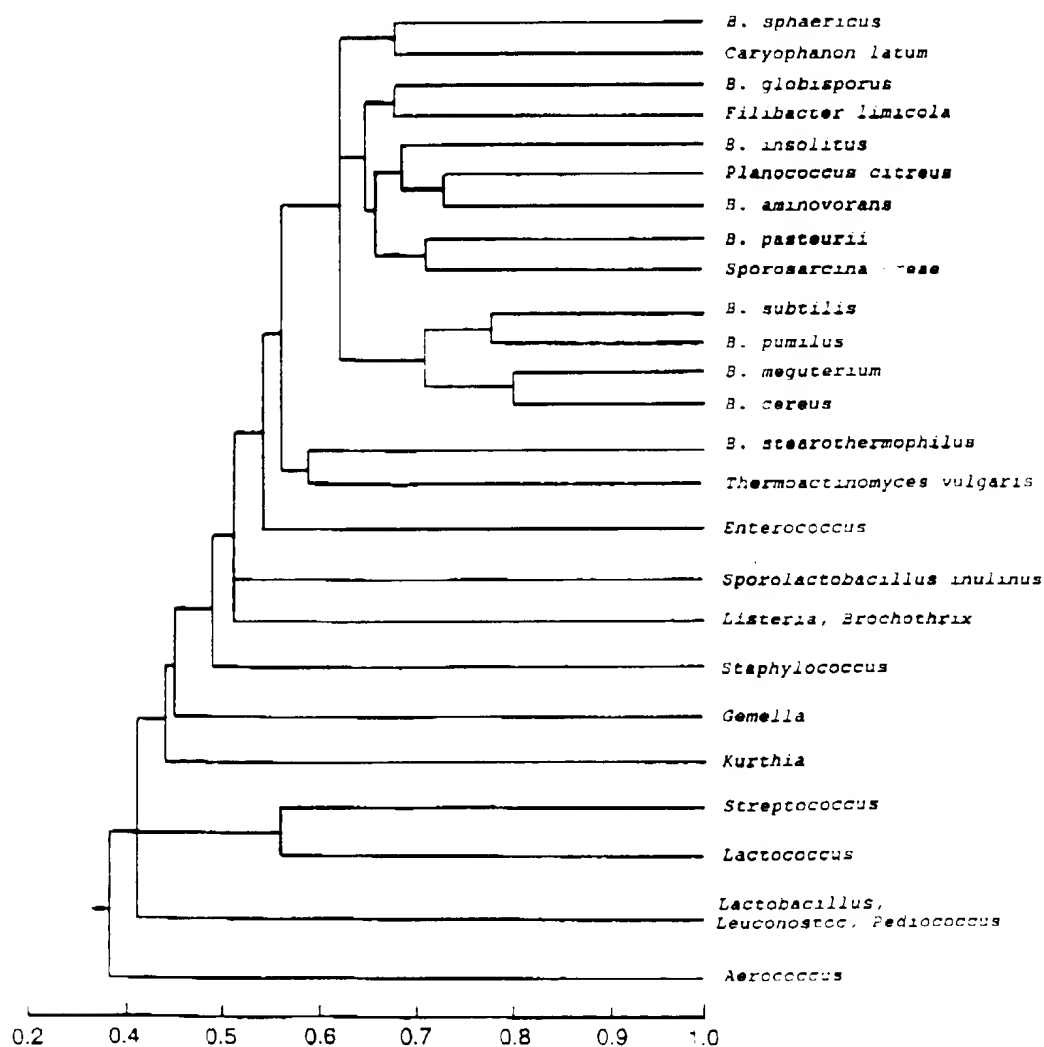


FIG. 4

PUTATIVE RHO-INDEPENDENT
TRANSCRIPTIONAL TERMINATION SITE

AAGCTGTCCGGTTTTTGC AAAAGTGGCTGTGACTGTATAAAAGAAATCGAAAAGACCGTTTTGGTGGTGAACCGGCTTTTTGGTTCCCTTTTAAACCAACTG 300

A V R F L U K W L .

End ORF4-5

*Clal**

σ^A

HpaI

HpaI

-35

-10

CGATAAATCGATCCCTTCTCTCTATTCACAGAACACAGGAGAGAAATATATATATCTAATTTGTTAACCTTTGAAATAAATTTGGTTAACTTTAGGTGAGAAG 400

POSSIBLE TRANSCRIPTIONAL REGULATORY SITE

Eco47III

RBS

BspIII

CGCTACACGTTCTTCAGTTATCAGTGAAGGGCGAGAAATGATGCAAGAGAGAACTTTTATAGTGTGAGAAATGAGGGCTTCAATGAAATGGATCTCATGA 500

M N Q E E T F Y S V R N R A S M N G S H E

Start *bioY*

FIG. 5A

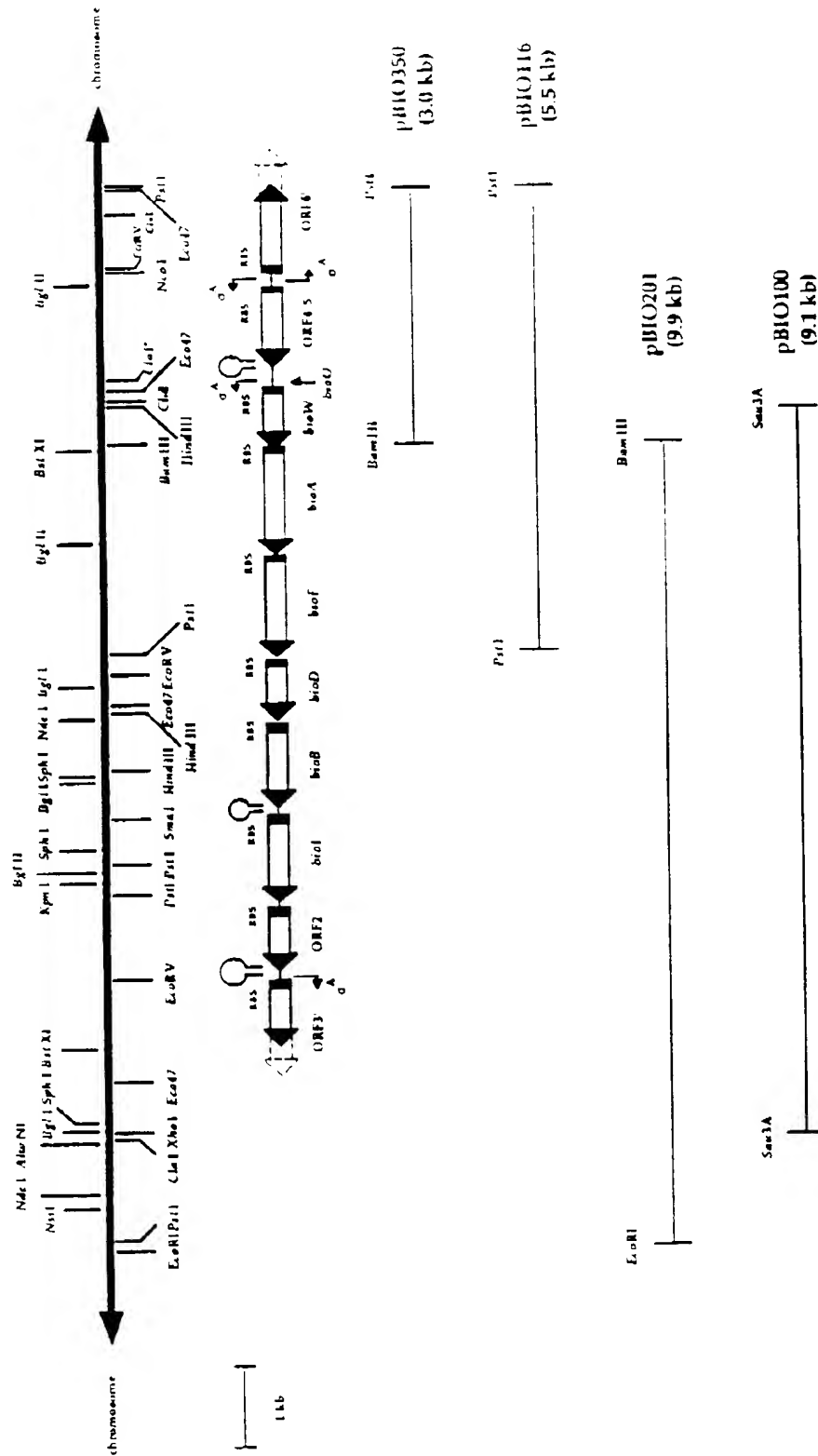


FIG. 5B

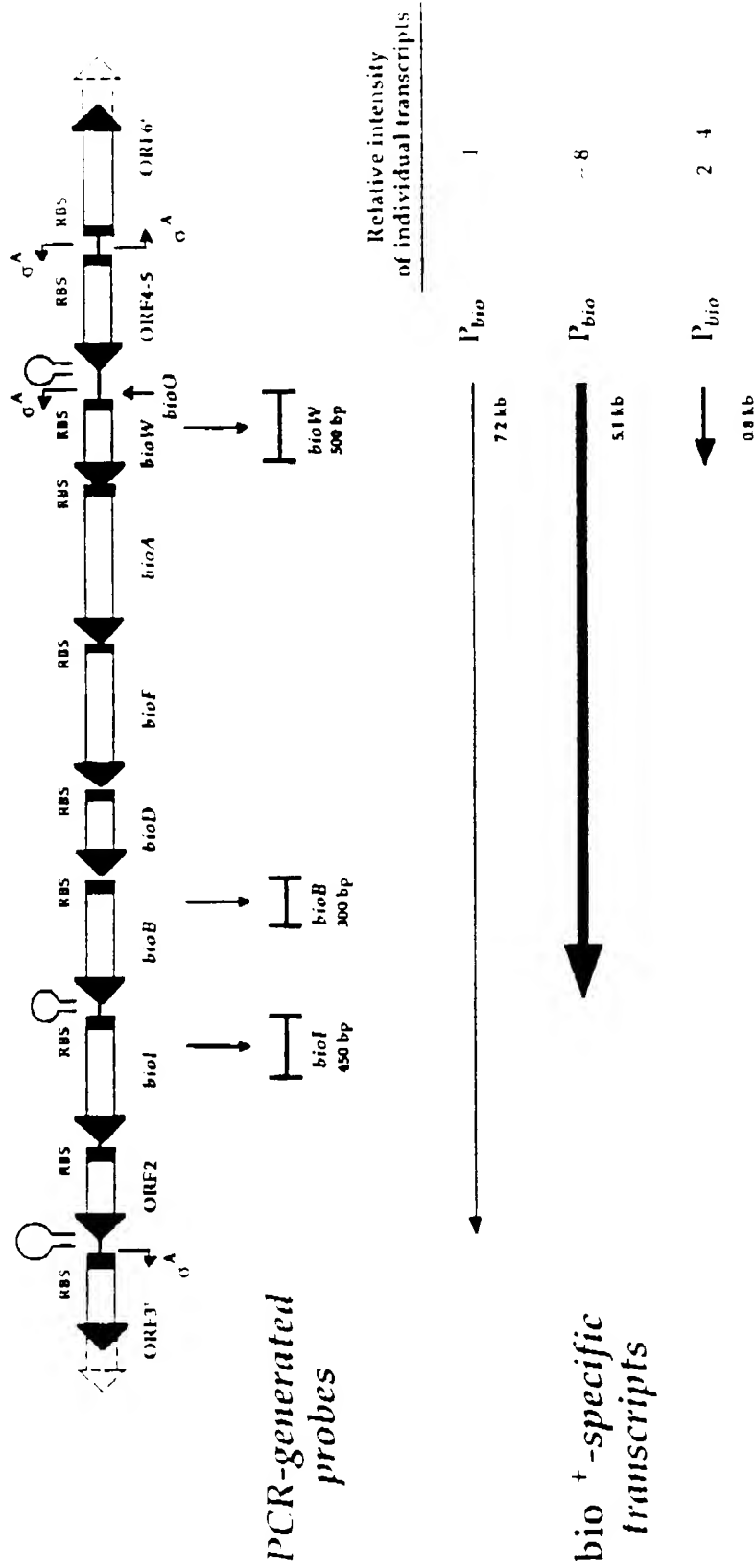
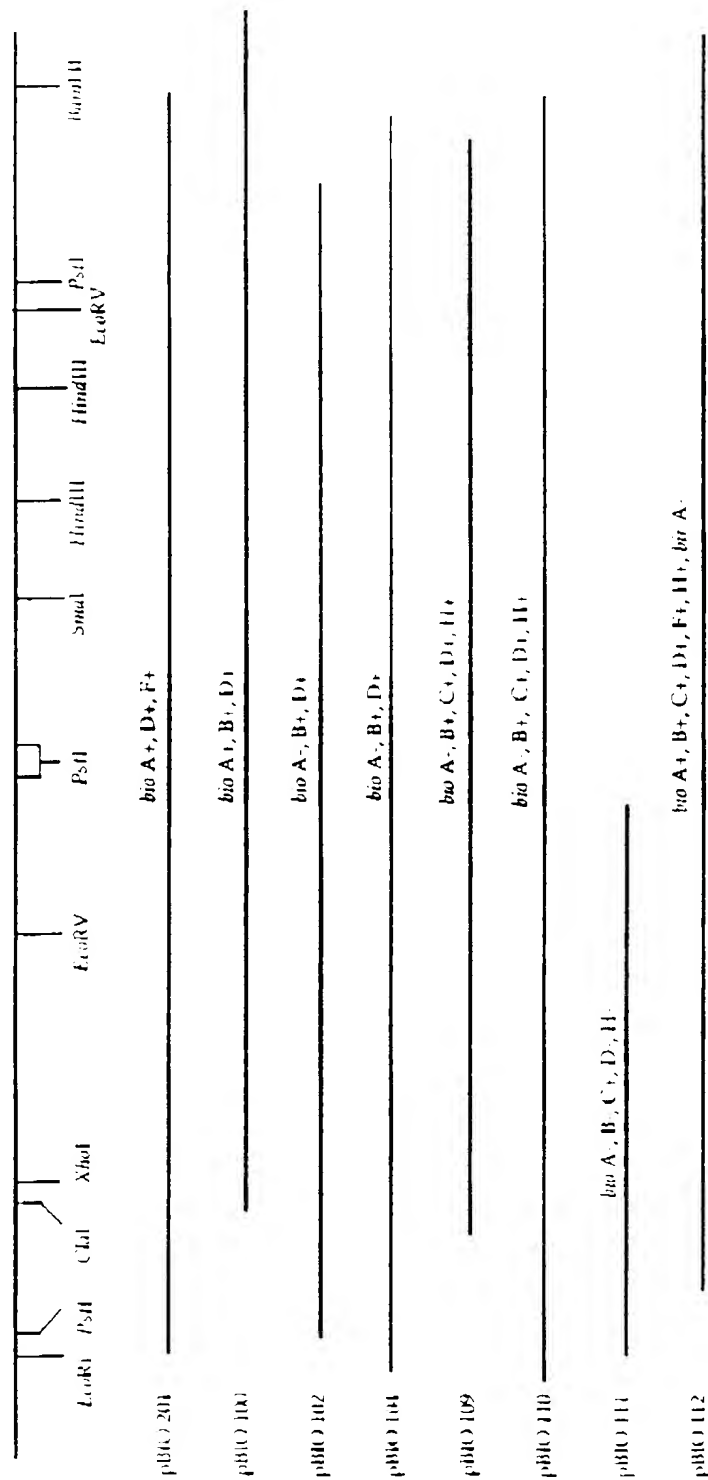
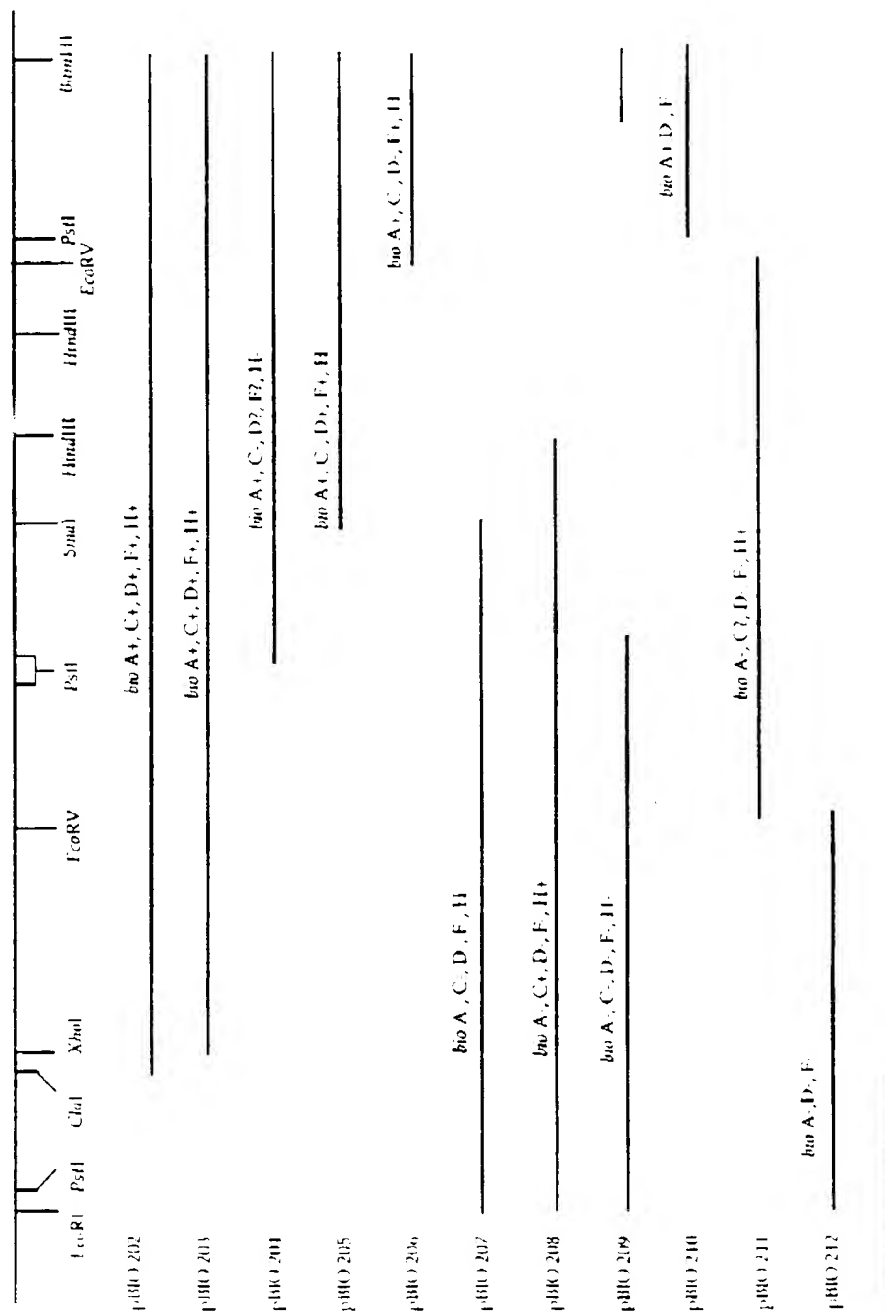


FIG. 6



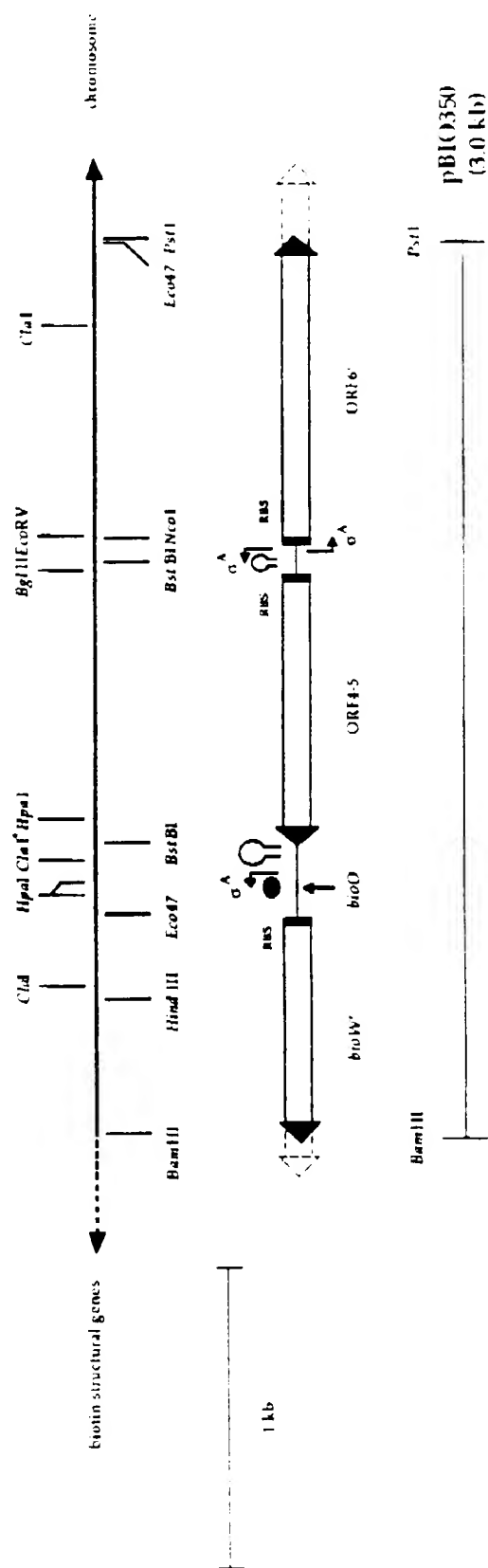
1 Complementation versus *E. coli bio* mutants. Mutants tested are indicated with a + (complementation) or - (no complementation). Mutants not listed were not tested.

FIG. 7

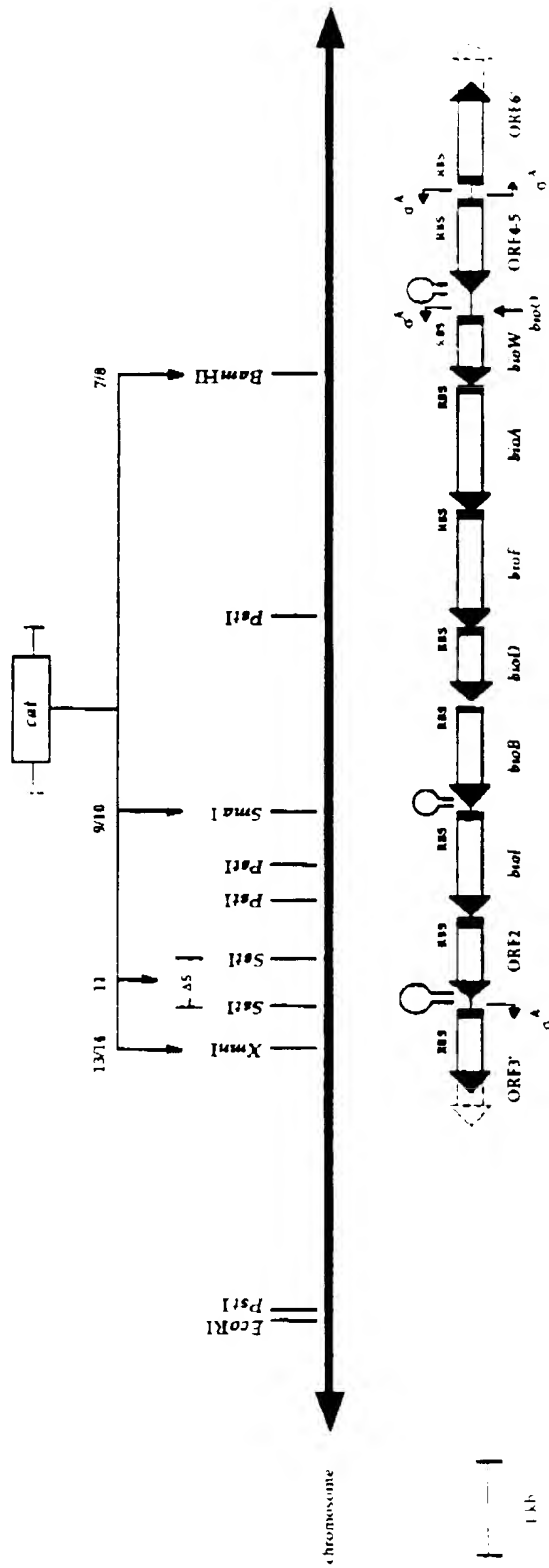


1. Complementation versus *E. coli* bio mutants. Mutants tested are indicated with a + (complementation), ? (partial complementation), or - (no complementation). Mutants not listed were not tested.

FIG. 8

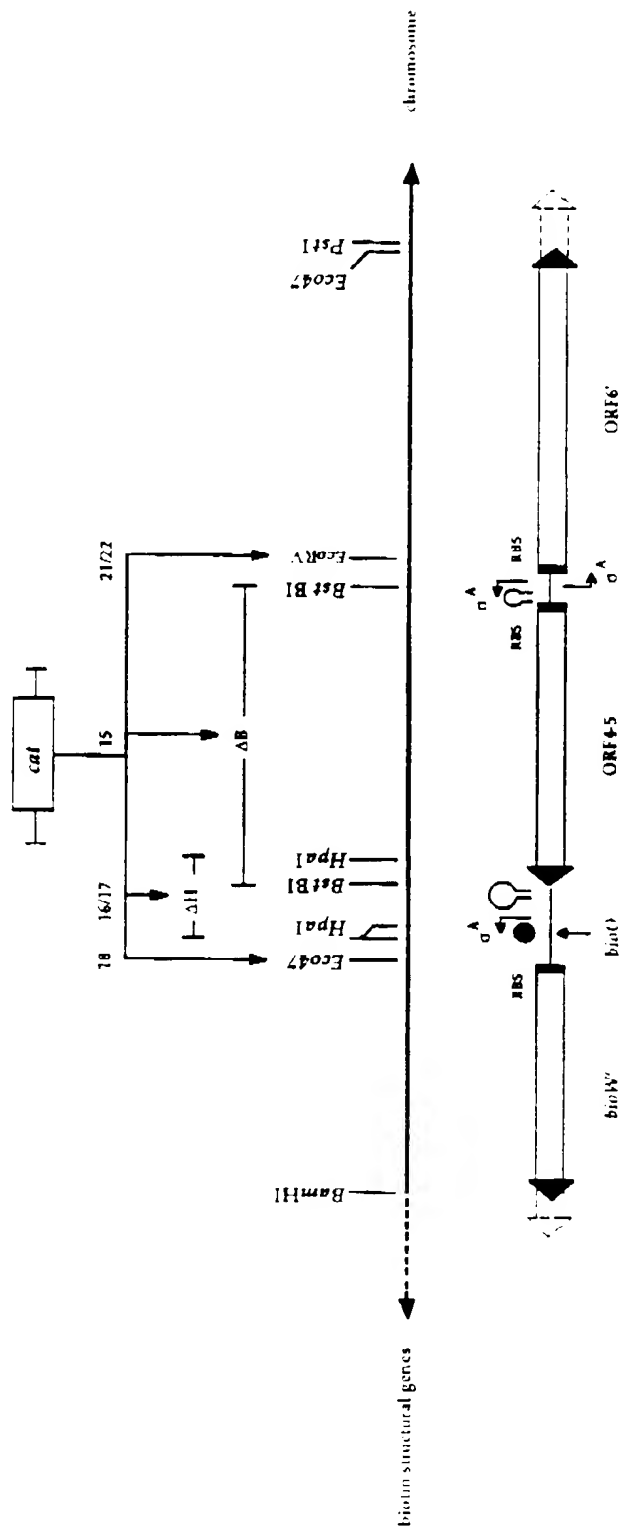


F/G. 9



<i>bio::cat</i> insertional mutation	<i>cat</i> orientation*	biotin phenotype	Growth on dethiobiotin	Growth on pimelic acid	Growth on minimal medium
<i>bioW::cat7</i>	—	—	—	—	—
<i>bioW::cat8</i>	+	—	+	—	—
<i>bioL::cat9</i>	—	+ / —	—	+	—
<i>bioL::cat10</i>	+	+ / —	—	+	—
ORF2::cat11	—	+	—	—	—
ORF3::cat13	+	+	—	—	+
ORF3::cat14	—	+	—	—	+

FIG. 10



<i>bio</i> cat insertional mutation	<i>cat</i> orientation ^a	Biotin phenotype	Growth on dethiobiotin	Growth on pimelic acid	Growth on minimal medium
ORF4- <i>cat</i> 15	+	+	—	—	+
ΔP_{bio} - <i>cat</i> 16	+	—	+	—	—
ΔP_{bio} - <i>cat</i> 17	—	—	—	—	—
P_{bio} - <i>cat</i> 18	+	—	+	—	—
ORF6- <i>cat</i> 21	—	+	—	—	+
ORF6- <i>cat</i> 22	+	+	—	—	+

FIG. 11

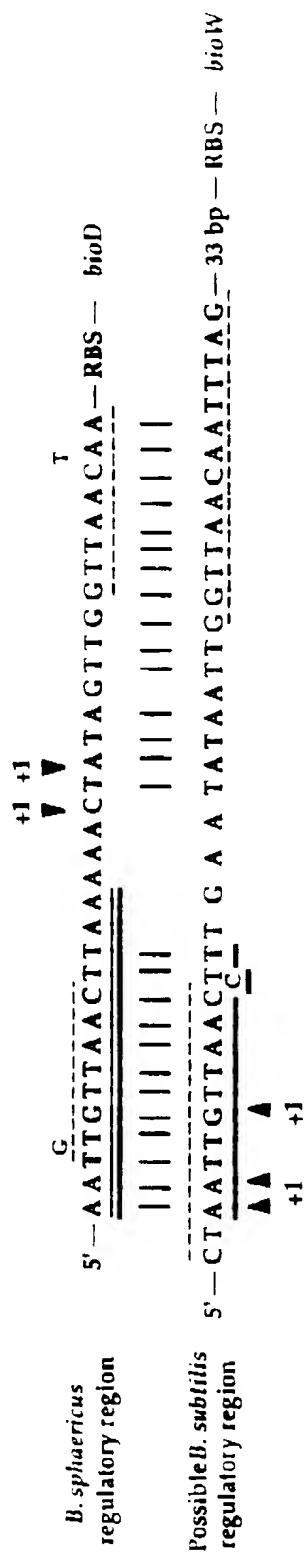


FIG. 12

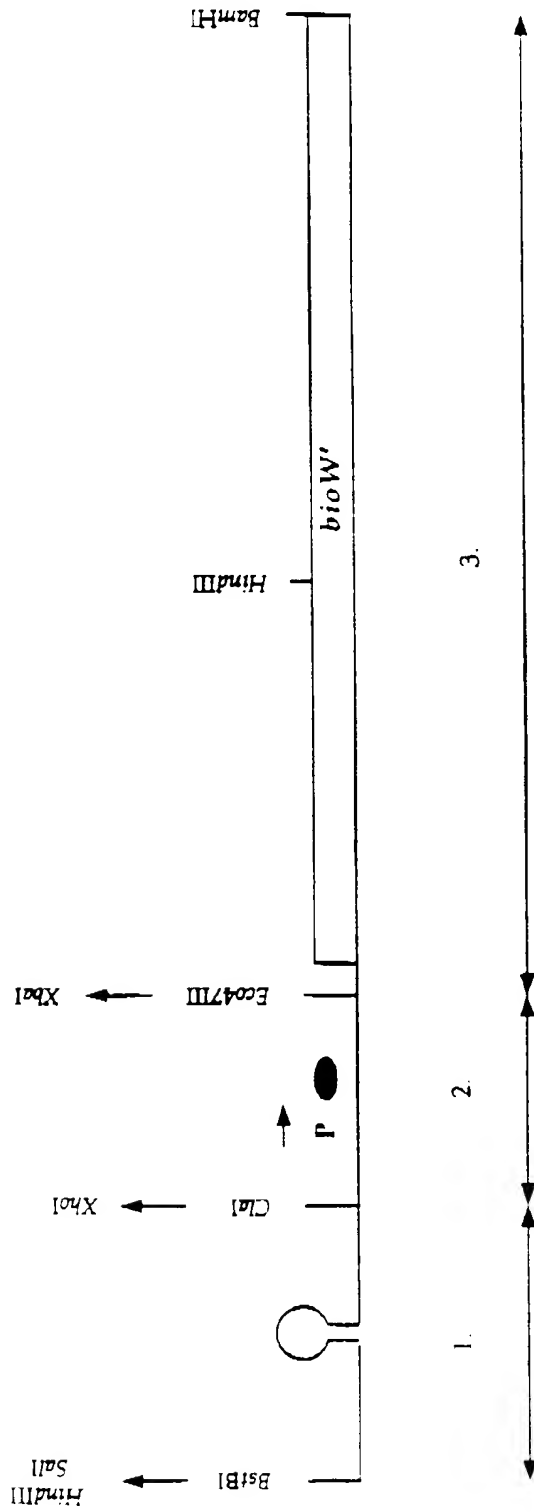
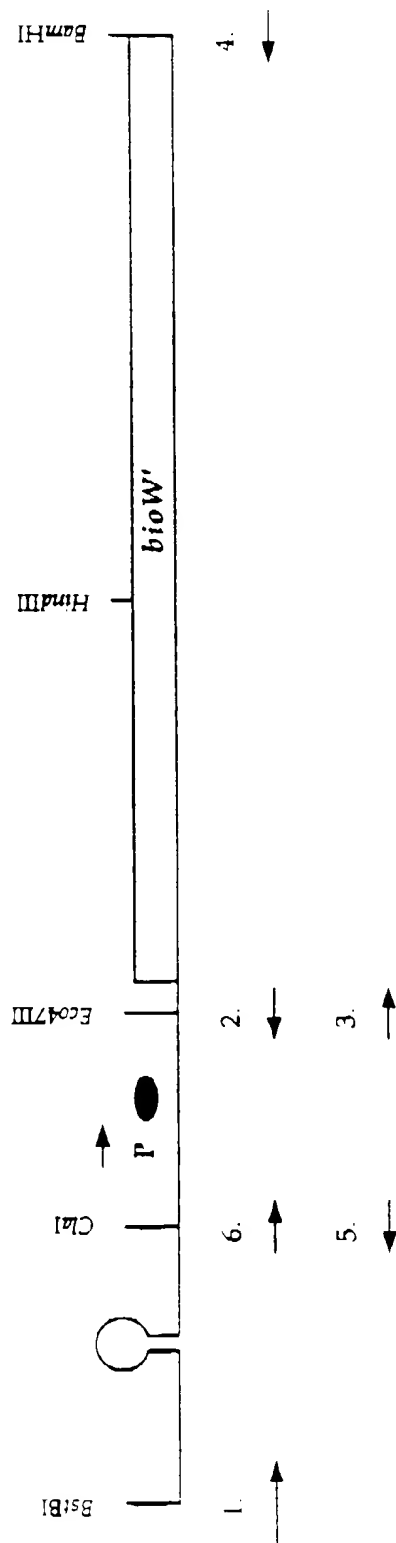


FIG. 13A



Number	Primer	Sequence	Comments
1	ORF4.1	5' - GGCCAAGCTGTGACCGAAACAGCAGTTATAAGGCAT - 3'	Mutates BstBI to HindIII SalI
2	BIOL5'	5' - GGCCCGTCTAGAGCTTCTCACCTA - 3'	Mutates Eco47III to XbaI
3	Leader1	5' - GGCCGAGAAAGCTCTAGACGTTCTTCAGTTATCAGT - 3'	Mutates Eco47III to XbaI
4	ANE1224	5' - CGCCAGGGTTTCCCGAGTCACGAC - 3'	Primes to vector pBIO350
5	BIOL3	5' - TAGAAGAAAGGCTCGAGTTATGGCAGTT - 3'	Mutates ClaI to XbaI
6	BIOL4	5' - AACTCCATAACTCGAGCCTTCTTCTA - 3'	Mutates ClaI to XbaI

FIG. 13B

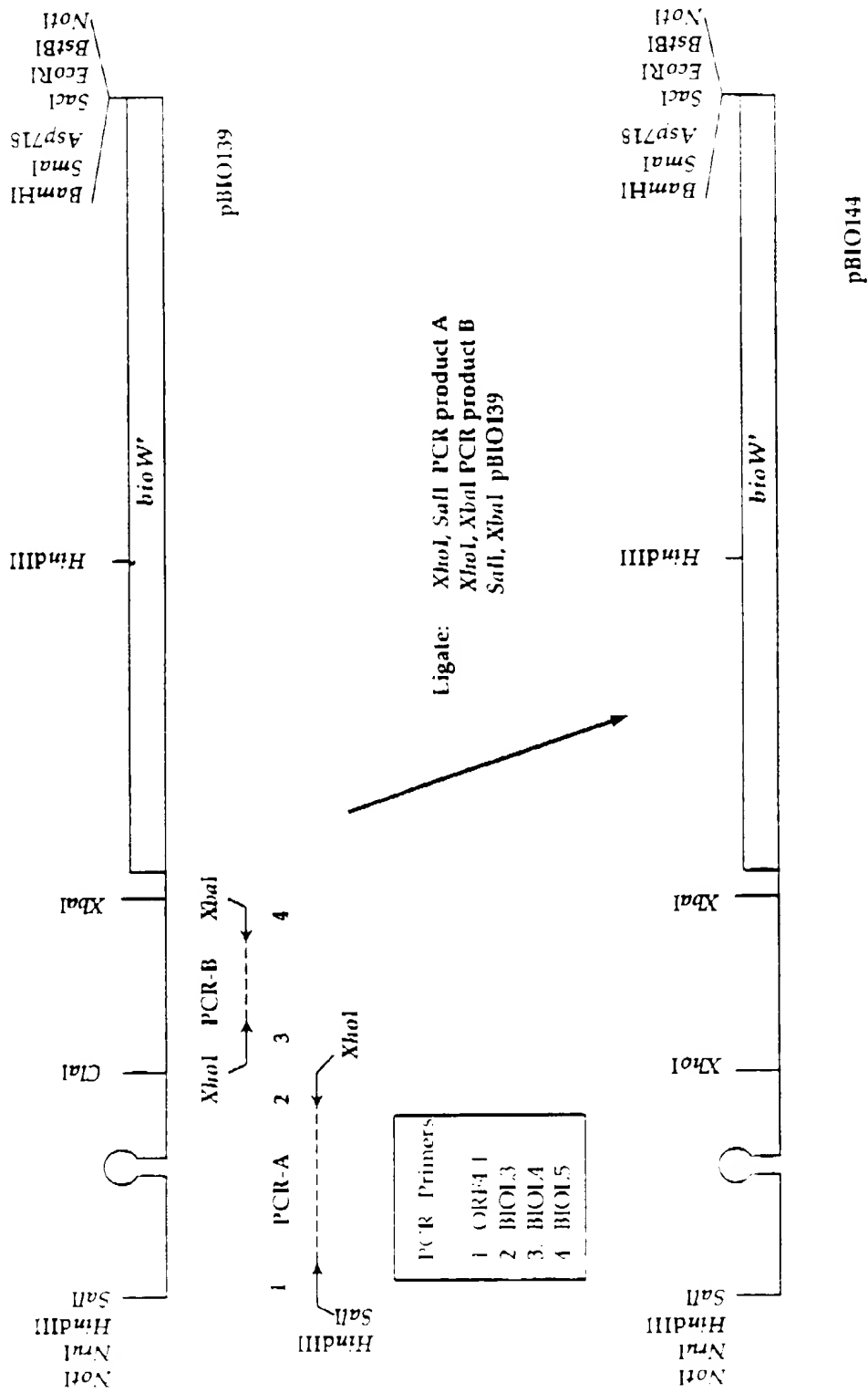


FIG. 14-1

```

1  CGCATCGGAG ATCCAAAGCC TGATCGCGCC GCGCCCGCAC CTTAGTCTTG
51  TTGGTGTACA CGATCGGTTA ACGCCGGCTG AGGGCGTGGA CAAAATCGAA
101 AAAGAATTGA CAGCTGTCTA TGCTGGACAG GGAGCTGCTG ATTGCTACCG
151 AGTGGTCCGT TCTGCTTCGG GACATTTCCA AACAGCAGTT ATAAGGCATG
201 AAGCTGTCCG GTTTTTGCAA AAGTGGCTGT GACTGTAAAA AGAAATCGAA
251 AAAGACCGTT TTGTGTGAAA ACGGTCTTTT TGTTTCCTTT TAACCAACTG
301 CCATAAATCG ATCCTTTCTT CTATTGACAG AAACAGGAGA GAATAATATA
351 TTCTAATTGT TAACCTTTGA ATATAATTGG TTAACAATTT AGGTGAGAAG
401 CGCTACACGT TCTTCAGTTA TCAGTGAAAG GGCGAGAAAT GATGCAAGAA
451 GAACTTTTTT ATAGTGTGAG AATGAGGGCT TCAATGAATG GATCTCATGA
501 AGACGGCGGA AAGCATATAT CCGGCGGAGA ACGGCTTATT CCTTCCATG
551 AGATGAAGCA TACAGTCAAT GCTTTATTAG AAAAAGGGTT ATCCCATTC
601 AGAGGAAAAC CTGATTTTAT GCAAATTCAA TTTGAAGAGG TACATGAATC
651 GATAAAAACC ATTCAGCCAT TGCCTGTGCA TACGAATGAA GTGAGCTGCC
701 CGGAAGAAGG ACAAAGCTT GCCCGATTGT TATTGAAAAA AGAAGGCGTT
751 TCACGAGACG TGATTGAAAA AGCATATGAA CAAATCCCTG AATGGTCAGA
801 TGTCAGGGGT GCGGTGTTGT TTGATATTCA TACAGGCAAG CGAATGGATC
851 AAACAAAAGA AAAAGGGGTG CGGGTCTCCA GAATGGATTG GCCGGACGCT
901 AATTTTGAAA AATGGGCGCT TCACAGTCAC GTGCCAGCTC ATTCAAGAAT
951 AAAAGAGGCC CTTGCGCTCG CTTCAAAGGT AAGCCGGCAC CCGGCAGTCG
1001 TTGCAGAATT ATGCTGGTCG GACGATCCGG ATTACATAAC AGGCTATGTT
1051 GCGGGTAAGA AAATGGGCTA TCAGCGTATT ACAGCAATGA AAGAATACGG
1101 GACTGAAGAG GGCTGCCGAG TCTTTTTTAT TGATGGATCC AATGATGTAA
1151 ACACGTACAT ACATGACCTG GAGAAGCAGC CTATTTTAAT AGAGTGGGAG

```

FIG. 14-2

1201 CAAGATCATG ACTCATGATT TGATAGAAAA AAGTAAAAAG CACCTCTGGC
 1251 TGCCATTTAC CCAAATGAAA GATTATGATG AAAACCCCTT AATCATCGAA
 1301 AGCGGGACTG GAATCAAAGT CAAAGACATA AACGGCAAGG AATACTATGA
 1351 CGGTTTTTCA TCGGTTTGGC TTAATGTCCA CGGACACCGC AAAAAAGAAC
 1401 TAGATGACGC CATAAAAAAA CAGCTCGGAA AAATTGCGCA CTCCACGTTA
 1451 TTGGGCATGA CCAATGTTCC AGCAACCCAG CTTGCCGAAA CATTAATCGA
 1501 CATCAGCCCA AAAAAGCTCA CGCGGGTCTT TTATTCAGAC AGCGGCGCAG
 1551 AGGCGATGGA AATAGCCCTA AAAATGGCGT TTCAGTATTG GAAGAACATC
 1601 GGGAAAGCCCG AGAAACAAAA ATTATCGCA ATGAAAAACG GGTATCACCG
 1651 TGATACGATT GGCGCCGTCA GTGTGGGTTT AATTGAGCTT TTTACCACG
 1701 TATACGGCCC GTTGATGTTT GAGAGTTACA AGGCCCCGAT TCCTTATGTG
 1751 TATCGTTCTG AAAGCGGTGA TCCTGATGAG TGCCGTGATC AGTGCCTCCG
 1801 AGAGCTTGCA CAGCTGCTTG AGGAACATCA TGAGGAAATT GCCGCGCTTT
 1851 CCATTGAATC AATGGTACAA GGCGCGTCCG GTATGATCGT GATGCCGGAA
 1901 GGATATTTGG CAGGCGTGCG CGAGCTATGT ACAACATACG ATGTCTTAAT
 1951 GATCGTTGAT GAAGTCGCTA CAGGCTTTGG CCGTACAGGA AAAATGTTTG
 2001 CGTGCGAGCA CGAGAATGTC CAGCCTGATC TGATGGCTGC CGGTAAAGGC
 2051 ATTACAGGAG GCTATTTGCC AATTGCCGTT ACGTTTGCCA CTGAAGACAT
 2101 CTATAAGGCA TTCTATGATG ATTATGAAAA CCTAAAAACC TTTTCCATG
 2151 GCCATTCCTA TACAGGCAAT CAGCTTGGCT GTGCGGTTGC GCTTGAANAAT
 2201 CTGGCATTAT TTGAATCTGA AAACATTGTG GAACAAGTAG CGGAAAAAAG
 2251 TAAAAAGCTC CATTTTCTTC TTCAAGATCT GCACGCTCTT CCTCATGTTG
 2301 GGGATATTCG GCAGCTTGGC TTTATGTGCG GTGCAGAGCT TGTACGATCA
 2351 AAGGAACTA AAGAACCCTA CCCGGCTGAT CGGCGGATTG GATACAAAGT
 2401 TTCCTTAAAA ATGAGAGAGT TAGGAATGCT GACAAGACCG CTTGGGGACG
 2451 TGATTGCATT TCTTCCTCCT CTTGCCAGCA CAGCTGAAGA GCTCTCGGAA
 2501 ATGGTTGCCA TTATGAAACA AGCGATCCAC GAGGTTACGA GCCTTGAAGA

FIG. 14-3

2551 TTGATTCTCTG GTTAAACGAG CCGTTAGACA GAATGAAAGA AGCCGGCGTA
 2601 CATCGTAACC TCGGGTCAAT GGATGGAGCG CCGGTTCCAG AGAGGAATAT
 2651 TGATGGCGAA AATCAAACGG TCTGGTCCTC AAACAATTAT TTAGGGCTCG
 2701 CAAGCCATAG ACGTTTGATC GATGCAGCCC AACAGCATT GCAGCAATTT
 2751 GGGACAGGAA GCAGCGGTTC ACGTTTAACG ACAGGCAATT CGGTCTGGCA
 2801 TGAAAAGCTA GAAAAGAAGA TTGCCAGCTT TAAACTGACA GAAGCGGCCC
 2851 TGCTGTTTTTC GAGCGGTAC TTGGCCAATG TCGGTGTCCT TTCATCCTTG
 2901 CCAGAAAAGG AAGATGTCAT TTTAAGTGAC CAGCTCAATC ATGCAAGTAT
 2951 GATCGACGGC TGCCGACTTT CTAAGGCTGA TACAGTTGTT TATCGGCATA
 3001 TTGATATGAA TGATCTTGAA AACAAAGCTGA ATGAAACACA GCGTTATCAG
 3051 CGCCGTTTTTA TCGTAACAGA CGGAGTATTC AGCATGGATG GCACAATCGC
 3101 CCCTCTTGAT CAGATCATCT CACTTGCAGAA ACGCTATCAT GCCTTCGTGG
 3151 TCGTTGATGA TGCCCACGCA ACAGGAGTTT TGGGCGATTC GGGACAAGGA
 3201 ACGAGTGAAT ACTTTGGTGT TTGTCCCGAC ATTGTTATCG GCACCTTAAG
 3251 CAAAGCTGTT GGCGCGGAAG GAGGTTTTTGC GGCAGGATCA GCGGTCTTCA
 3301 TCGACTTTTTT GCTGAACCAT GCCAGAACAT TTATCTTTCA AACCGCTATT
 3351 CCGCCAGCCA GCTGTGCGGC TGCTCAGAG GCTTTCAACA TCATTGAAGC
 3401 CAGCAGGGAA AAACGACAGC TTTTATTTTC TTATATCAGC ATGATCAGAA
 3451 CCAGTCTGAA GAATATGGGT TATGTGGTGA AAGGAGATCA CACACCGATT
 3501 ATTCCTGTAG TCATTGGCGA TGCCCATAAA ACGGTCCTAT TTGCTGAAAA
 3551 ACTGCAGGGC AAGGGAATTT ATGCTCCTGC CATTCGGCCG CCAACCGTTG
 3601 CGCCGGGTGA AAGCCGATT CGAATTACAA TCACGTCTGA CCACAGTATG
 3651 GGTGATATTG ATCATTTGCT GCAAACATTT CATTCATCG GAAAGGAGCT
 3701 GCACATCATT TGAGGGGTTT TTTTGTGACG GGAAGTATA CAGAAGTAGG
 3751 GAAAACGGTT ATATCCAGCG GTCTTGCTGC CTTATTGAAA GACAATAATA
 3801 GACATGTCGG GGTGTATAAA CCATTTTTAA GCGGGATATC GCGCCATCAT
 3851 CCAGATAGTG ATACAAGTTT GCTGAAAGAT ATGTCGCAGA CCAGTCTTTC

FIG. 14-4

3901 TCATGAAGAC ATTACGCCTT TTGCCTTCAA GCGCGCGCTT GCACCATACG
 3951 TTGCAGGGAA ACTTGAGGGA AAGACTGTCA CCATGGAAGA GGTPTTAAGC
 4001 CATTGGGGGC GGATTAGAGA AAAACATGAA TGCTTCATCG TAGAAGGTGC
 4051 AGGCGGTATT TCTGTGCCAT TGGGAGAGGA CTATTTGGTC AGTCATGTCA
 4101 TAAAAGCGTT GCAGCTTCCC ATGATTATTG TGGCGCGTCC TCGCCTTGGG
 4151 ACCATTAATC ATACCTTTTT AACTGTCAAA TATGCAGAAA GCATGGGGCT
 4201 TCCAATCGCC GGAATTATCA TCAATGGAAT CAGTGA CTCT CCTGATGAAG
 4251 ATGAAAAAAC CAATCCTGAG ATGATTGAGC GCTTATGCGG TGTGCGGATT
 4301 TTAGGGGTTA CGCCAAAGCT TGCCAAACGTG ACGAAAGAAA CGGTTCTACA
 4351 TATGGTAAAA GACCATATCA ATCTATCATT ACTGATGAAT CAAGTGGGGG
 4401 TATGAGAATG AATCAATGGA TGGAACTCGC AGACCGGGTG CTGGCTGGAG
 4451 CAGAAGTGAC TGACGAAGAG GCGCTTTCAA TATTACATTG TCCTGATGAA
 4501 GATATTTTGC TATTAATGCA CGGGGCTTTT CACATCAGAA AACACTTTTA
 4551 CGGAAAAAAA GTAAAGCTCA ATATGATTAT GAATGCGAAA TCCGGGCTCT
 4601 GCCCGGAAAA CTGCGGCTAT TGTTACAGT CTGCGATTTC GAAAGCGCCG
 4651 ATTGAGTCTT ACCGGATGGT GAATAAGGAA ACGCTGCTTG AAGGCGCGAA
 4701 GCGGGCGCAC GATCTGAATA TCGGCACATA TTGTATCGTG GCAAGCGGCA
 4751 GAGGTCCGTC TAACAGAGAA GTGGATCAGG TCGTAGATGC GGTTCAGGAA
 4801 ATTAAAGAGA CGTATGGACT GAAGATTTGT GCATGTCTTG GACTGTTGAA
 4851 GCCAGAGCAG GCGAAGCGGC TCAAAGATGC AGGAGTAGAC CGCTATAATC
 4901 ATAATTTGAA TACGTCACAG AGAAACCATT CAAACATCAC AACCTCACAT
 4951 ACATACGATG ACAGAGTCAA TACGGTTGAA ATCGCAAAAG AATCGGGGCT
 5001 GTCTCCGTGT TCAGGCGCCA TTATCGGGAT GAAGGAGACG AAACAGGATG
 5051 TCATTGACAT CGCCAAAAGC TTGAAGGCTC TTGACGCGGA TTCCATTCTT
 5101 GTGAATTTTT TGCATGCAAT TGATGGCAGC CCGTTAGAAG GCGTCAACGA
 5151 ATTAACCCCG CTGTATTGTT TAAAAGTGCT GCGCTGTTC CGTTTTATCA
 5201 ATCCATCAAA AGAAATTGCG ATTTCCGGAG GAAGAGAGGT CAATCTCCGC

FIG. 14-5

5251 ACATTGCAGC CATTAGGGCT TTACGCCGCA AACTCCATTT TTGTCGGAGA
 5301 CTACTTAACA ACTGCCGGGC AAGAGGAGAC GGAGGATCAT AAAATGCTGA
 5351 GTGATTTAGG CTTTGAAGTT GAATCAGTCG AAGAAATGAA GGCTAGTTTA
 5401 AGTGCGAAAA GCTGAAAGAA TCAATAAAAG CAATCGGTAT GATGTCGATT
 5451 GTTTTTATTT TTGAACAGAA AGGAGAAAAAT CACGTGACAA TTGCATCGTC
 5501 AACTGCATCT TCTGAGTTTT TGAAAAACCC ATATTCTTTT TACGACACAT
 5551 TGGGAGCTGT TCATCCTATC TATAAAGGGA GTTTCCTTAA ATACCCGGGC
 5601 TGGTATGTCA CAGGATATGA AGAAACGGCT GCTATTTTGA AAGATGCGAG
 5651 ATTCAAAGTC CGCACCCCGC TGCCTGAGAG CTCAACCAA TATCAGGACC
 5701 TTTACATGT GCAAATCAA ATGATGCTGT TTCAGAACCA GCCTGATCAT
 5751 AGACGATTGC GGACGCTTGC CAGCGGAGCG TTTACGCCGA GAACGACAGA
 5801 GAGTTATCAG CCGTATATCA TTGAAACTGT CCATCATTTG CTTGATCAAG
 5851 TGCAAGGTAA AAAAAAGATG GAGGTCATTT CGGACTTTGC TTTTCCTTTA
 5901 GCAAGTTTTG TCATAGCTAA CATTATAGGT GTACCGGAGG AAGATAGGGA
 5951 GCAATTAAAG GAGTGGGCTG CGAGTCTCAT TCAAACGATT GATTTTACCC
 6001 GCTCAAGAAA GGCATTAACA GAGGGCAATA TTATGGCTGT GCAGGCTATG
 6051 GCATATTTCA AAGAGCTGAT TCAAAGAGA AAACGCCACC CTCAACAGGA
 6101 TATGATCAGC ATGCTCTTGA AGGGGAGAGA AAAGGATAAG CTGACGGAAG
 6151 AGGAGGCGGC ATCTACGTGC ATATTGCTGG CGATCGCCGG ACATGAGACA
 6201 ACGGTCAATC TCATCAGCAA TTCAGTCCTT TGTCTGCTGC AGCATCCAGA
 6251 ACAGCTTTTG AAAGTGAGAG AAAATCCAGA TCTTATTGGT ACCGCAGTCG
 6301 AGGAATGTTT ACGCTATGAA AGCCCCACGC AAATGACAGC CAGAGTTGGC
 6351 TCAGAGGATA TTGACATCTG CGGGGTGACG ATCCGTCAAG GAGAACAAGT
 6401 CTATCTTTTG TTAGGAGCGG CTAATCGAGA CCCTAGCATA TTCACGAACC
 6451 CCGATGTCTT CGATATTACG AGAAGTCCTA ATCCGCATCT TTCATTGGG
 6501 CATGGCCATC ATGTTTGCTT AGGGTCCTCG CTGGCACCAT TAGAAGCGCA
 6551 AATTGCGATT AACACTCTTC TGCAGCGAAT GCCCAGCCTT AATCTTGCGG

FIG. 14-6

6601 ATTTTGAATG GCGGTATCGG CCGCTTTTGT GATTTCCGGC GCTTGAGGAG
 6651 CTGCCCGTGA CTTTGAATA AGCCTAAGAA TGTGAGTGCC AAAAAAGTGT
 6701 CAGCCCCGCC GAAAATGGGC AATCTATAAA AAAGGGGAGT GAACATCGTG
 6751 AAAAAAGTGC TGATCGCCGG CGGAAATGGT GTGATTGGGA GACTGCTTGC
 6801 TGAAGGGCTT ATTTGAGACT ATGAAGTGAC TGTGCTTGAT AAAGATCATT
 6851 TCGATGGCAA AGCCTCTTCC ATTCAGGCTG ACGCGGCAAA TTATGAGGAG
 6901 CTGTTGAAGA AGATTCCAAA AGATACCGAT GCCATCTTGA ATTTACTCGC
 6951 TGTGAAAATC AAATACGATA TTATGGACAT CGCTGAGTTT GAAAAATGA
 7001 CGGATGTTTT CTATAGGGCA AGCTATTATC TGTGCCGTGC GGCAGCGGAG
 7051 CTCGGCATTG AAAAGCTCGT GTTCGCCAGC AGCAATCATG TCACAGATGT
 7101 ATATGAAAAA GACGGGCGCT CGCTCTTAGG ACGGGAAATC ACAACAAGCG
 7151 ATTATCCGCT GTCAAAAAAC TTGTACGGTG TATTAAAGCT GACCTCTGAA
 7201 CAGATCGGCC ATTTGTTTTA TTTGGAAAAT AAGCTATCAG TAATCAACCT
 7251 TCGAATCGGA ACAGTCGTGA CAGATGAAAT GGATACGCTG CATGAAAAAG
 7301 AACGGACGAA AAAGACACTG CTTTCTCACC CCGATCTGCT GTCGATTTTC
 7351 AAAGCCGCCA TTGAGACCAA CATCCGGTAT GGCACCTATT ACGCGCTCTC
 7401 TGATAATCCG GGCCGGCCAT GGTCCATTGA ATCTGCCGTG AATGAACTTG
 7451 GGTTTTCGCC ACAATCAAT ACGGCTGAAC TTCTGAACGA GGAGGAGAAC
 7501 GGAGCATAAT CATTTTCTAA GATTATGCTC TTTTCTTTT GTTATCGGTC
 7551 TCAATTCGCG GCAGCCCCCG CCGGCGCGG GACACTGTTC AAATGATTAT
 7601 AGACATGGCA ATCACAGATT TGCTACATTT TAGACACGAT ATCGTCACAT
 7651 GCTGAGCTCG GTTTCAAAA ATATGATAAC GCTTACAAAG GGAGGTGGGA
 7701 GCTATCGCAC ATTCAGTCAA AAACCGTCTG TTTGATATGT TGATTTATGG
 7751 TTTCTTGCTG ATGTTGCTT TAATATGCGT ACTTCCGTTC ATTCATGTTA
 7801 TCGCAGCATC CTTTGCCACA GTAGAAGAAG TCGTGTGAA AAAATTTATT
 7851 TTAATACCGA CCACTTTTTC GCTAGATGCT TATCGCTACA TTTTTCAC
 7901 AGATATTATT TATAAGAGTT TGCTTGTTTC TGTGTTTGTG ACAGTGATAG

FIG. 14-7

7951 GCACTGCGGT CAGCATGTTT CTTTCGTCAC TGATGGCTTA CGGGTTATCC
8001 CGCCGTGATT TAATCGGCCG GCAGCCGCTC ATGTTTCTCG TCGTATTTAC
8051 GATGCTGTTT AGCGGCGGCA TGATTCCGAC TTTCCCTGTG GTCAAATCGC
8101 TTGGATTGCT CGATTCTTAC TGGGCGCTTA TTTTGCCGAC AGCCATTAAT
8151 GCCTTTAACC TGATCATTCT GAAAACTTC TTTCAAATA TCCCGTCAAG
8201 CCTGGAAGAG TCCGCGAAAA TTGACGGGTG CAATGATCTG GGCATATTCT
8251 TTAAAATTGT GCTGCCGCTG TCTCTTCCTG CGATCGCAAC GATTTCACTA
8301 TTTTATGCGG TCACGTATTG GAACACGTAT ATGACAGCGA TCTTGTAATT
8351 AAATGATTCA GCAAAATGGC CAATTCAGGT GCTTCTGCGC CAAATCGTCA
8401 TTGTATCAAG CGGTATGCAG GGGGATATGT CTGAAATGGG GTCCGGGCAGC
8451 CCGCCGCCTG AGCAAACCAT NNNNNTGG

FIG. 15

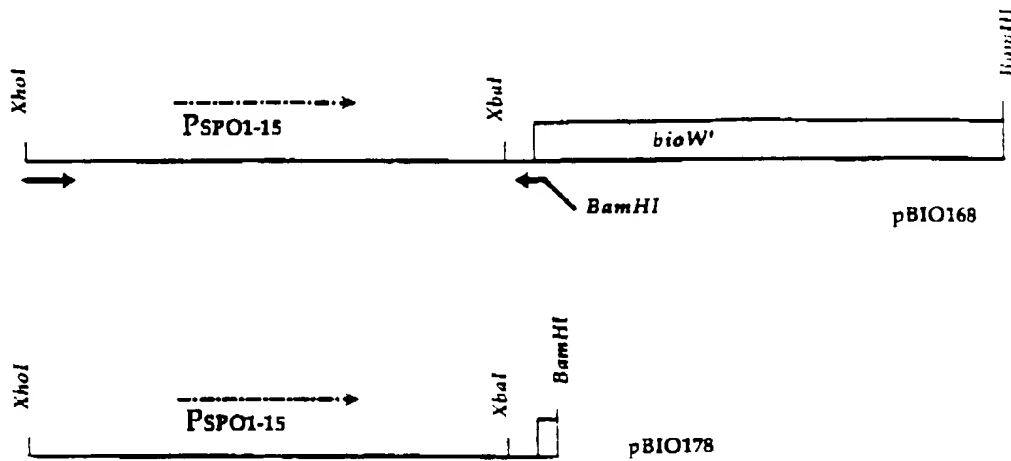
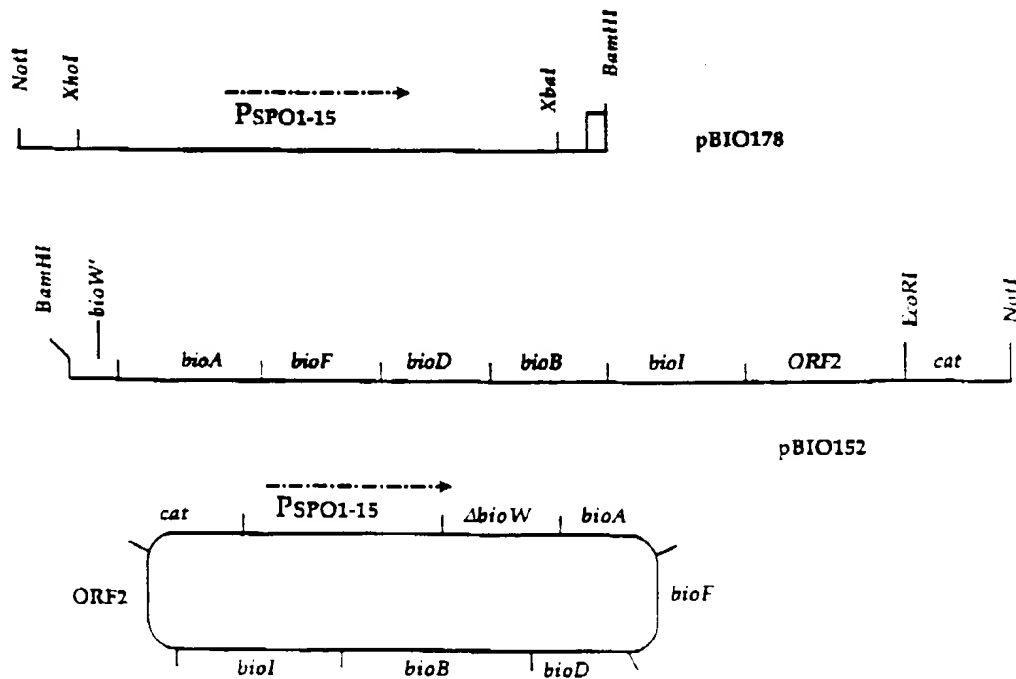
Bioautography of Biotin and Vitamin Standards and Fermentation Broths



Lane	Sample
1	DAPA, 150 ng
2	Fermentation B38 (30 hr), 1 μ l of a 10 fold dilution
3	Fermentation B39 (30 hr), 1 μ l of a 10 fold dilution
4	Fermentation B40 (30 hr), 1 μ l of a 10 fold dilution
5	D-thiobiotin, 10 ng
6	Biotin, 10 ng

FIG. 16

A. Generation of pBIO178 by PCR

B. Ligation of pBIO178 and pBIO152 to generate SPO1-15-*bio*(Δ *bioW*)

BamHI

Nucleotides: N - any nucleotide, R - purine, W - weak, i.e. A or T.

FIG. 17B

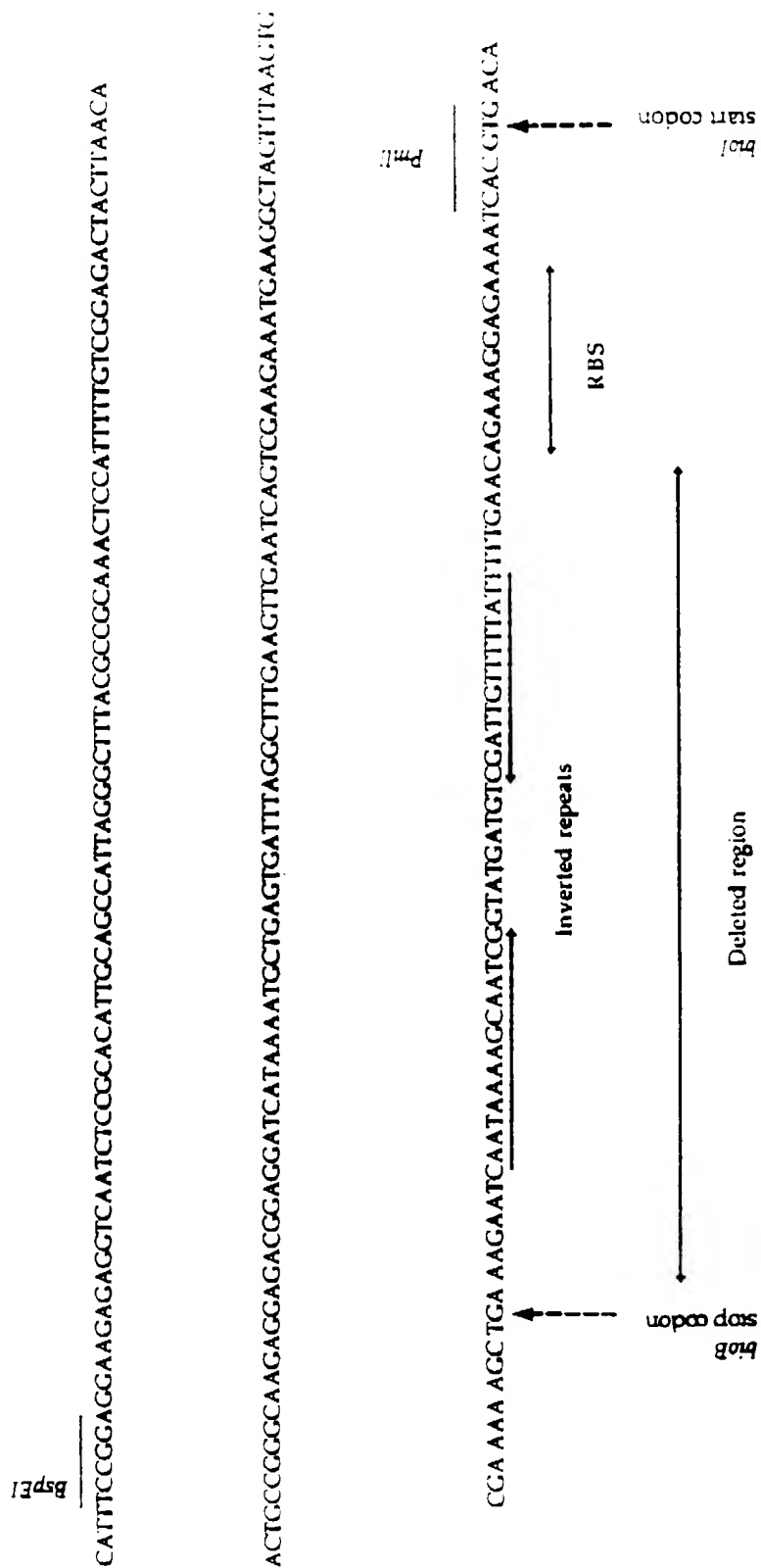


FIG. 18

Azelaic acid resistance of PA3 and PA6.

